REMARKS

I. Sequence Listings

This is in response to the Office Action dated October 19, 2005.

By the above amendment, the sequence listings referred to at page 106 of applicants' specification, i.e., SEQ ID NO:1 and SEQ ID NO:2, have been added to this application as separate pages at the end of the specification.

Except for the inclusion of the serial numbers of this application and the applications from which this application claims priority, these sequence listings are identical to the sequence listings that appeared at pages 64-69 of applicants' provisional application number 60/219,529 (the '529 application) from which this application claims priority under 35 USC §119(e). The '529 application was incorporated by reference in the present application (see page 2, lines 3-8, of applicants' specification), and thus this amendment does not introduce new matter into this application.

Pursuant to 37 CFR §1.821(e), submitted herewith is a copy of SEQ ID NO:1 and SEQ ID NO:2 in computer readable form. Pursuant to 37 CFR §1.821(f), the undersigned hereby states that the computer readable form is identical to the written sequence listings.

II. The October 19th Office Action

In the October 19th Office Action, the Examiner indicated that:

(1) Claims 187 and 208 would be allowable if written in independent form.

(2) Claims 186, 199, 201-203, 205-207, and 217-222 are rejected under 35 USC §102(b) as alleged anticipated by PCT Patent Publication No. WO 99/20589 (Roufogalis et al.) or PCT Patent Publication No. WO 99/09140 (Julius et al.).

(3) Claims 200 and 204 are rejected under 35 USC §103(a) as allegedly unpatentable over Roufogalis et al. or Julius et al.

Applicants respectfully traverse the Examiner's rejections for the following reasons.

A. **The §102 Rejections**

In the paragraph bridging pages 2-3 of the Office Action, the Examiner wrote:

> Roufogalis et al. teach the use of phenylalkanols (ginerol analogues) in the treatment or prophylaxis of pain by action on sensory nerves and/or through anti-inflammatory action (page 1, lines 1-10). Note on page 20, lines 8-38 states the gingerol analogues are effective as capsaicin receptor antagonists. Note page 21, lines 1-10 shows the analogues are effective in the treatment of pain and inflammatory conditions.

It is true that at page 20, lines 16-19, of their patent publication, Roufogalis et al. do use the word "antagonise", but as we will now show, that use is not the same as Roufogalis et al. saying that their gingerol analogues are "capsaicin receptor antagonists" as called for by applicants' claims. Rather, Roufogalis et al.'s use of this word is, in fact, part of their showing that their gingerol analogues are capsaicin receptor agonists, not antagonists.

Capsaicin and other capsaicin receptor agonists have the property that in addition to activating capsaicin receptors, they also desensitize them. As explained in applicants' specification at page 4, lines 10-25:

> The capsaicin receptor's channel opens in response to elevated temperatures (higher than about 45° C). Capsaicin and related compounds ... are stimuli that lower the threshold [for] channel opening, so that in the presence of any of these stimuli the capsaicin receptor can be opened even at room temperature.
>
> Opening of the capsaicin receptor channel is followed by the release of inflammatory peptides from neurons expressing the receptor and other nearby neurons, increasing the pain response. After initial activation by capsaicin the capsaicin receptor undergoes a rapid desensitization, possibly via phosphorylation of intracellular sites of the receptor. Capsaicin and related VR1 agonist vanilloid compounds have enjoyed long-pharmaceutical use as topical anaesthetics. While such compounds initially cause a strong burning sensation, receptor desensitization provides pain relief. (emphasis added)

As also discussed in applicants' specification (see page 5, lines 6-7), resiniferatoxin (RTX) is a potent and specific agonist of the capsaicin receptor. Studies

with this compound have shown that like capsaicin, it exhibits activation first followed by desensitization. Moreover, capsaicin and RTX exhibit cross-desensitization. As described in a 1993 article entitled "Resiniferatoxin -- An Ultrapotent Capsaicin Analogue":[1]

> The twin hallmarks of capsaicin action are the spectrum of induced responses and the desensitization to repeated capsaicin administration. As observed for capsaicin, resiniferatoxin treatment leads to desensitization to subsequent resiniferatoxin application. Consistent with resiniferatoxin and capsaicin sharing a common site of action, desensitization by either compound leads to cross-desensitization to the other.

See also a 1997 article from the same group entitled "Differential activation and desensitization of sensory neurons by resiniferatoxin" (Exhibit B hereto) which states:

> As observed for capsaicin, after initial excitation, RTX treatment also leads to desensitization to subsequent RTX application; in addition, desensitization by either compound leads to cross-desensitization to the other.[2]

With this background in mind, it becomes immediately evident that when Roufogalis et al. use the word "antagonise" at page 20 and elsewhere in their patent publication, they are actually describing "cross-desensitization" between capsaicin

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[1] The full cite for the article is: Blumberg PM, Szallasi A, Acs G (1993) Resiniferatoxin, an ultrapotent capsaicin analogue. In: Capsaicin in the study of pain (Wood JN, ed), pp 45-62. London: Academic. A copy of the article is attached as Exhibit A. The above quotation appears at page 50.

[2] The full cite for this article is: Acs G, Biro T, Acs P, Modarres S, Blumberg, P (1997) Differential activation and desensitization of sensory neurons by resiniferatoxin. J. Neurosci. 17:5622-5628. The above quotation appears at page 5622.

Additional discussions of desensitization of the capsaicin receptor can be found in the following references, copies of which are attached as Exhibit C: (1) Szallasi A, Blumberg PM (1990) Minireview: Resiniferatoxin and its analogs provide novel insights into the pharmacology of the vanilloid (capsaicin) receptor. Life Sci 47:1399-1408. (2) Szallasi A, Blumberg PM (1993) Mechanisms and therapeutic potential of vanilloids (capsaicin-like molecules). Advances in Pharmacology 24:123-155. (3) Szallasi A, Blumberg PM (1993) [^{3}H]resiniferatoxin binding by the vanilloid receptor: species-related differences, effects of temperature and sulfhydryl reagents. Naunyn-Schmiedeberg's Arch Pharmacol 347:84-91.

agonists, not the action of a capsaicin receptor antagonist. Thus, the specific passage of Roufogalis et al. cited by the Examiner reads as follows:

> A synthetic gingerol analogue was found to antagonise the effect of capsaicin, and *vice versa*, in rat mesenteric artery bed." (Roufogalis et al. at page 20, lines 16-19.)

The "and *vice versa*" is the tip-off that cross-desensitization is being described, not the action of a capsaicin receptor antagonist. Thus, based on the "*vice versa*", this sentence could equally have read:

> Capsaicin was found to antagonise the effect of a synthetic gingerol analogue, and *vice versa*, in rat mesenteric artery bed."

Plainly, this would not have been a teaching that capsaicin is a capsaicin receptor antagonist since it is the agonist for which the receptor is named. Likewise, the sentence as written in the reference is not a teaching that synthetic gingerol analogues are capsaicin receptor antagonists.

The remainder of the Roufogalis et al. reference supports the fact that the reference does not disclose capsaicin receptor antagonists. For example, the table on pages 63-64 of the reference, as well as Roufogalis et al.'s "conclusion" number 1 at page 65, disclose that 11 out of the 14 gingerol analogues tested had the ability to open ion channels which are permeable to extracellular Ca^{2+}, just like capsaicin opens such channels. Indeed, the reference's preferred compound -- 1-(4-hydroxy-3-methoxyphenyl) dodecan-3-ol [3.93] (a vanilloid compound; hereinafter referred to as the "[3.93] compound") -- achieved peak intracellular calcium levels as a result of channel opening similar to those achieved with capsaicin, i.e., 661±376 nM for the [3.93] compound versus 824±122 nM for capsaicin for the "high responses" and 187±66 nM versus 134±78 for the "low responses". This opening of ion channels permeable to extracellular Ca^{2+} is, of course, a well-recognized characteristic of capsaicin receptor agonists (see, for example, the discussions of calcium uptake in the references of Exhibits A-C hereto).

Roufogalis et al.'s detailed discussion of the [3.93] compound at page 22, line 31 to page 23, line 5, clearly demonstrates that the compound has capsaicin-like agonist, rather than antagonist effects. Thus, the effects of this gingerol analog were blocked by pretreatment with capsaicin, and the effects of capsaicin were blocked by pretreatment with the analog, results that are inconsistent with the actions of a capsaicin receptor antagonist, but are completely consistent with the desensitization effects of a capsaicin receptor agonist. Furthermore, the effects of the [3.93] compound were blocked by the (then putative, now archetypical) capsaicin receptor antagonist capsazepine (Roufogalis et al. at page 22, line 38 – page 23, line 3). In fact, Roufogalis et al. explicitly state at page 24, lines 12-13: "In summary, these results suggest that 1-(4-hydroxy-3-methoxyphenyl)dodecan-3-ol [3.93] acts like capsaicin..." Thus, the disclosure of Roufogalis et al. provides compounds that exhibit capsaicin agonist properties, in sharp distinction from the compounds specified in the pending claims.

Finally, attached hereto as Exhibit D is a copy of a 2002 article in the British Journal of Pharmacology whose authors include all of the named inventors of the Roufogalis et al. PCT publication cited by the Examiner. The article is entitled "Gingerols: a Novel Class of Vanilloid Receptor (VR1) Agonists" and contains the following abstract:[3]

> 1 Gingerols, the pungent constituents of ginger, were synthesized and assessed as agonists of the capsaicin-activated VR1 (vanilloid) receptor.
>
> 2 [6]-Gingerol and [8]-gingerol evoked capsaicin-like intracellular Ca^{2+} transients and ion currents in cultured DRG neurones. These effects of gingerols were blocked by capsazepine, the VR1 receptor antagonist.
>
> 3 The potency of gingerols increased with increasing size of the side chain and with the overall hydrophobicity in the series.

[3] The full cite for this article is: Dedov VN, Tran VH, Duke CC, Conner M, Christie MJ, Mandadi S, Roufogalis B (2002) Gingerols: a novel class of vanilloid receptor (VR1) agonists. Br. J. Pharmacol. 137:793-798.

> 4 We conclude that gingerols represent a novel class of naturally occurring VR1 receptor agonists that may contribute to the medicinal properties of ginger, which have been known for centuries. The gingerol structure may be used as a template for the development of drugs acting as moderately potent activators of the VR1 receptor. (emphasis added)

[8]-gingerol, referred to in this abstract, is one of the compounds studied in the PCT publication and found to open ion channels which are permeable to extracellular Ca^{2+} (see the table at pages 63-64 of Roufogalis et al. discussed above). Plainly, and not surprisingly given their data, Roufogalis et al., the authors of the Examiner's reference, understood that their gingerol analogues function as agonists, not antagonists. This being the case, the Examiner's assertion that the Roufogalis et al. reference anticipates applicants' claims, which unequivocally call for the use of a "capsaicin receptor antagonist", cannot stand and should be withdrawn.[4]

The Examiner's second reference -- the Julius et al. reference -- is even further from applicants' claims than Roufogalis et al. This reference discloses and discusses cloned polynucleotides encoding capsaicin receptor proteins, i.e., it is concerned with the receptor, not with agonists or antagonists of the receptor. In particular, page 11, lines 1-10, of Julius et al. relied on by the Examiner read:

> The polynucleotides of the invention can also be used as a molecular probe with which to determine the structure, location, and expression of capsaicin receptor, receptor subtypes, and capsaicin receptor-related polypeptides in mammals (including humans) and to investigate potential associations between disease states or clinical disorders (particularly those involving acute and chronic pain or inflammation) and defects or alterations in receptor structure, expression, or function.

The polynucleotides of this passage are neither receptor agonists or antagonists, and are clearly outside the scope of applicants' claims. Moreover, although the

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[4] Further evidence from another lab that gingerol analogues function as agonists of the capsaicin receptor can be found in Someya A, Horie S, Yamamoto H, Murayama T (2003) Modifications of capsaicin-sensitive neurons in isolated guinea pig ileum by [6]-gingerol and lafutidine. J Pharmacol Sci 92:359-366, a copy of which is attached as Exhibit E.

reference mentions that its cloned receptors might be used to study agonists or antagonists, it does not disclose or suggest the methods and/or capsaicin receptor antagonists that are the specific subject of applicants' pending claims. As such, the reference does not constitute an anticipation of those claims.

B. The §103 Rejections

The Examiner also cited the Roufogalis et al. and Julius et al. references against Claims 200 and 204, in this case under §103. Each of these claims ultimately depends on independent Claim 201. They add the further requirement that:

> a dose of the capsaicin receptor antagonist that is five times the minimum dose needed to provide analgesia in an adult mammalian laboratory animal, in an animal model for determining pain relief, does not cause sedation when administered to an adult mammalian laboratory animal in an animal model assay of sedation, wherein the same species is used in assessing analgesia and sedation.

In the October 19th Office Action, the Examiner characterized these claims as requiring that "the capsaicin antagonist is administered five times the minimum dose needed to provide analgesia in an adult mammalian" animal and then asserted that the claims would have been obvious to a person of ordinary skill in the art because one "would have expected to increase the dose amount to accommodate the size of the adult mammal...." (10/19/05 Office Action at pages 4-5.)

Although the claims do refer to administering "a dose at five times the minimum dose needed to provide analgesia in an adult mammalian laboratory animal," this administration is not an administration in general, but rather is an administration in the context of a sedation assay. Thus, the claims require that the 5X administration "does not cause sedation when administered to an adult mammalian laboratory animal in an animal model assay of sedation."

As discussed above, neither Roufogalis et al. nor Julius et al. disclose or suggest the subject matter of independent Claim 201. That being the case, they certainly do not disclose or suggest a capsaicin receptor antagonist that has the low sedation properties

called for by dependent Claims 200 and 204. Accordingly, applicants respectfully submit that the Examiner's §103 rejection is unfounded and should be withdrawn.

There are further differences both with regard to lack of anticipation and non-obviousness between applicants' claims (dependent and independent) and the cited references. However, a detailed discussion of those differences is not considered necessary at this point in view of the fundamental differences discussed above.

III. Conclusion

Based on the foregoing, applicants believe that their application is now in condition for allowance. Accordingly, reconsideration and the issuance of a Notice of Allowance for the application are respectfully requested.

Respectfully submitted,

Date: 1/19/06

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Exhibit A

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CHAPTER 3

RESINIFERATOXIN – AN ULTRAPOTENT CAPSAICIN ANALOGUE

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3.1 Background

Natural toxins have afforded extraordinarily powerful tools for the identification and characterization of biochemical and physiological pathways. The toxins combine two advantages. First, they are preselected for important processes within the target organism, since interaction with their target has to cause a sufficiently dramatic response for the toxin to be of use to the producing organism. Second, the toxins tend to have high potency, since high potency is more efficient for the producing organism, whereas the endogenous ligand for the target site of the toxin may have a lower affinity more suitable for rapid physiological

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CAPSAICIN IN THE STUDY OF PAIN
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Resiniferatoxin is a tricyclic diterpene of the resiniferonol series (Figure 1). It was initially isolated by Hergenhahn, Adolf and Hecker from the fresh latex of *Euphorbia resinifera* (Adolf *et al.*, 1982; Hergenhahn *et al.*, 1975), where it was found in an abundance of 0.26%. It was independently isolated by Evans and coworkers from the latex of *Euphorbia poissonii* (incorrectly described as *E. poisonii*) (Schmidt and Evans, 1975; Evans and Schmidt, 1976). Three properties of resiniferatoxin were unusual for a phorbol-related diterpene. First, it showed a more rapid and transient response than did typical phorbol esters such as phorbol 12-myristate 13-acetate. Whereas phorbol 12-myristate 13-acetate has similar inflammatory potency at 3 h and at 24 h in the mouse ear assay, resiniferatoxin displayed no inflammatory activity at the latter time (Hergenhahn *et al.*, 1982). Second, the absolute inflammatory potency of resiniferatoxin was orders of magnitude greater than that of phorbol 12-myristate 13-acetate, the most potent of the typical phorbol esters. Third, its structure activity relations were atypical. Extensive analysis has indicated that a free C-20 hydroxyl group is required for biological activity of the typical phorbol esters (Hecker, 1978). This requirement is now understood from the computer modelling, which suggests that the free C-20 hydroxyl group corresponds to the free C-3 hydroxyl group of the endogenous analogue *sn*-1,2-diacylglycerol (Wender *et al.*, 1986). In contrast, the extraordinary irritant activity of resiniferatoxin was attributable to the *p*-hydroxy, *m*-methoxyphenylacetate ester at C-20 (Adolf *et al.*, 1982; Schmidt and Evans, 1979).

Further biological investigation dating back a decade strongly supported the interpretation that the primary site of action of resiniferatoxin was different from that of the typical phorbol esters. Resiniferatoxin showed no activity when assayed for tumour promotion (Hergenhahn *et al.*, 1984). This conclusion is corroborated by the observation that resiniferatoxin fails to induce hyperplasia in mouse skin (Hergenhahn *et al.*, 1984; confirmed by Szállási and Blumberg, 1989b); chronic hyperplasia is though to represent a good predictor of activity for mouse skin tumour promoters (Sisskin *et al.*, 1982). We showed that resiniferatoxin failed to induce the responses typical of the phorbol esters in chicken embryo fibroblasts and in mouse 3T3 cells, viz. morphological change, induction of deoxyglucose uptake, and loss of fibronectin (Driedger and Blumberg, 1980b). Resiniferatoxin likewise failed to induce EB viral antigens, another typical phorbol ester response (Zur Hausen *et al.*, 1979). Upon identification of phorbol ester receptors, direct binding studies confirmed that resiniferatoxin only bound to the phorbol ester receptors with low affinity (Driedger and Blumberg, 1980a; Delclos *et al.*, 1980). These results have been confirmed in more recent studies using partially purified protein kinase C (Szállási *et al.*, 1989b).

The biological studies of more than a decade ago thus strongly argued for the existence of a distinct target for resiniferatoxin possessing high affinity and involved in erythema. The structure activity studies had focused attention on the C-20 ester as the structural feature of resiniferatoxin responsible for its unique activity. This group is analogous to the active moiety of capsaicin (Figure 1).

The structure activity studies for capsaicin further enhance this analogy, since Szolcsányi had demonstrated that both the inverse amide of capsaicin and the corresponding ester retained good potency in the rat eye-wiping assay (Szolcsányi and Jancsó-Gábor, 1975). On the other hand, for research groups working in the vanilloid field, the analogy was perhaps obscured since the initial structure reported both by Hecker's group (Hergenhahn *et al.*, 1975) and by Evans' group (Schmidt and Evans, 1975) was incorrect – the initial reports described the side chain as a *m*-methoxy *m*-hydroxyphenylacetate ester and the correct structure was only reported in the body of a subsequent paper (Adolf *et al.*, 1982). Demonstration that resiniferatoxin indeed functions as a high affinity analogue of capsaicin was provided by this laboratory, first for induction of hypothermia in mice (de Vries and Blumberg, 1989) and then for the three responses of neurogenic inflammation, hypothermia and chemogenic pain in rats (Szállási and Blumberg, 1989a). Subsequent analyses by this laboratory and by others have greatly expanded on these initial reports (see Szállási and Blumberg (1990b, 1993b) for reviews). Our current perspective on resiniferatoxin action and the vanilloid pathway as revealed by resiniferatoxin are described below.

3.2 Biological responses to resiniferatoxin

3.2.1 Comparisons with capsaicin

3.2.1.1 Excitation

Qualitatively, resiniferatoxin has been found to induce a generally similar pattern of responses as observed for capsaicin. The compounds differ, however, in their relative potencies for different responses. In initial studies, induction of neurogenic inflammation and hypothermia by resiniferatoxin occurred at three to four orders of magnitude lower doses than for capsaicin (Szállási and Blumberg, 1989a). In contrast, induction of chemogenic pain, assessed in the rat eye-wiping assay, showed only slightly greater potency for resiniferatoxin than for capsaicin (Szállási and Blumberg, 1989a). Moreover, not all responses were mimicked by resiniferatoxin. In the rat, resiniferatoxin was unable to induce the pulmonary chemoreflex triad of apnoea, hypotension and bradycardia, although resiniferatoxin did induce these responses in the cat (Szolcsányi *et al.*, 1990). The biological systems examined for resiniferatoxin responses and the comparative potencies for resiniferatoxin and capsaicin are summarized in Szállási and Blumberg (1990b, 1993).

The basis for the differences in pattern of response to resiniferatoxin and capsaicin remains to be established. As described below, we favour the hypothesis that multiple classes of receptors exist with differential selectivity between resiniferatoxin and capsaicin. The hypothesis that the demonstrated differences

in pharmacokinetics may manifest themselves as differences in potency also deserves serious consideration.

3.2.1.2 Desensitization

The twin hallmarks of capsaicin action are the spectrum of induced responses and the desensitization to repeated capsaicin administration. As observed for capsaicin, resiniferatoxin treatment leads to desensitization to subsequent resiniferatoxin application. Consistent with resiniferatoxin and capsaicin sharing a common site of action, desensitization by either compound leads to cross-desensitization to the other. The biological systems examined and the relative potencies for desensitization by resiniferatoxin and capsaicin are summarized in Szállási and Blumberg (1990b, 1993).

The acute effects of resiniferatoxin (Section 3.2.1.1) demonstrate that it is not simply a more potent version of capsaicin. Perhaps reflecting its relatively poor ability to induce the pulmonary chemoreflex, resiniferatoxin shows a much greater therapeutic index for desensitization than does capsaicin. Thus, in adult rats the maximum tolerated dose of resiniferatoxin administered subcutaneously (s.c.) is 100 times the ED_{50} for desensitization of either hypothermia or neurogenic inflammation (Szállási and Blumberg, 1989a). For capsaicin, in contrast, the ED_{50} is the maximum tolerated dose and a schedule of multiple, incremental doses of capsaicin is required for complete desensitization (Szállási and Blumberg, 1989a).

It is clear that the potency for desensitization by resiniferatoxin is not the same for all responses. In particular, chemogenic pain, which is differentially insensitive to induction by resiniferatoxin, is differentially sensitive to resiniferatoxin. Thus, whereas the ED_{50} for desensitization of hypothermia or neurogenic inflammation is 5×10^{-6} g/kg s.c., the ED_{50} for desensitization of chemogenic pain is 1×10^{-7} g/kg s.c. (Szállási and Blumberg, 1989a). This difference in potency was confirmed when the same group of animals was assessed for each response.

The same conclusion, that different responses possess different susceptibilities for desensitization, is also supported by the kinetics of recovery from desensitization (Section 3.2.4). No recovery of chemogenic pain is found at times at which most of the neurogenic inflammatory response has been restored (Szállási *et al.*, 1989a).

Treatment of neonatal rats with resiniferatoxin (300 μg/kg) leads to long-lasting inhibition of chemogenic pain and to marked loss of neurogenic inflammatory response (Szállási *et al.*, 1990), as observed previously for capsaicin.

3.2.1.3 Cellular effects

Resiniferatoxin functions as an ultrapotent, selective capsaicin analogue not only at the whole animal and tissue levels but also, and not unexpectedly, at the cellular level. This latter analysis has been persuasively carried out by Winter

et al. (1990). Resiniferatoxin induced Ca^{2+} influx into cultured dorsal root ganglion cells from newborn rats with approximately 200-fold greater potency than did capsaicin. Only a subset of cells responded to resiniferatoxin with increased membrane conductance; this subset of cells was the same as the subset responsive to capsaicin. The reversal potential for resiniferatoxin and for capsaicin was the same, arguing for a similar effect on conductance.

The biological studies demonstrate that resiniferatoxin and capsaicin act on the same class of neurones to induce similar responses. The analysis of resiniferatoxin binding (Section 3.3.1) demonstrates directly that the site of action is identical for resiniferatoxin and capsaicin.

3.2.2 Effect of resiniferatoxin on neuronal morphology and neuropeptides

Capsaicin treatment causes long-lasting ultrastructural changes in the B-neurones of the dorsal root ganglia of the rat. Similar changes were observed at 24 h in rats treated with resiniferatoxin (100 μg/kg) (Szolcsányi *et al.*, 1990) or in rats treated with 300 μg/kg resiniferatoxin and examined either at 4 h or two weeks (Szállási *et al.*, 1989a).

Cumulative resiniferatoxin treatment with a total dose of 1.5 mg/kg caused marked loss of calicitonin gene-related peptide (CGRP) both from the perikarya of neurones of the dorsal root and Gasserian ganglia and from the nerve terminals in the dorsal horn of the spinal cord (Szolcsányi *et al.*, 1990). Depletion of CGRP was also observed from both dorsal root and Gasserian ganglia and from the dorsal horn of the spinal cord of adult rats treated with resiniferatoxin as neonates (Szállási *et al.*, 1990).

Resiniferatoxin caused release of CGRP from guinea-pig lung (Franco-Cereceda *et al.*, 1990; Lou *et al.*, 1991). The effective concentration was 0.3 nM, compared to a concentration of 10 nM for capsaicin. The rate of release was slower than for capsaicin, and, as described in other systems, ruthenium red inhibited release induced either by resiniferatoxin or capsaicin. Resiniferonol 9,13,14-orthophenylacetate, the resiniferatoxin analogue lacking the homovanillyl ester, failed to cause release. In guinea-pig heart resiniferatoxin treatment also led to release of CGRP-LI (Franco-Cereceda *et al.*, 1991). Release was inhibited by ruthenium red.

3.2.3 Structure activity relations

The different spectrum of relative potencies for resiniferatoxin and capsaicin proves that pharmacology can dissect patterns of response through the vanilloid pathway. The limited structure activity analysis conducted so far on vanilloids of the resiniferonol series, termed 'resiniferanoids' in analogy with capsaicinoids, affords a glimpse of rich opportunities. We have examined in some detail three analogues (Figure 2). Tinyatoxin, initially isolated from *E. poissonii*, differs from

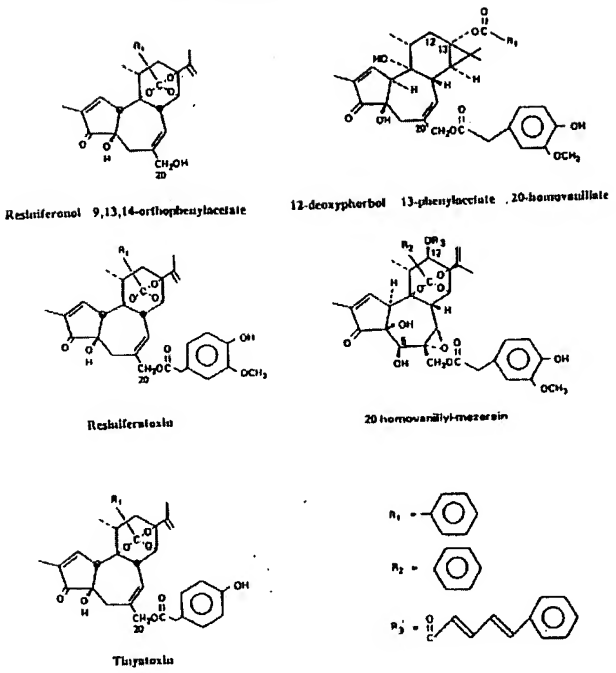

Figure 2

resiniferatoxin in being a *p*-hydroxyphenylacetate ester rather than a homovanillate ester. It is six-fold less irritant than resiniferatoxin in the mouse ear reddening assay (Schmidt and Evans, 1979); two-fold less potent for producing hypothermia in the mouse (Szállási *et al.*, 1991); similar for producing oedema in the ear of the rat (Szállási *et al.*, 1991); and similar for binding to the vanilloid receptors in the rat dorsal root ganglion membranes (Szállási *et al.*, 1991; see below). In contrast, tinyatoxin was almost inactive in inducing an eye-wiping response in the rat and mouse (Szállási *et al.*, 1991). The results with tinyatoxin corroborate the findings with resiniferatoxin that pungency has different structural requirements than neurogenic inflammation and hypothermia. They suggest, moreover, that tinyatoxin may represent the compound of choice for dissection of these two classes of response.

12-Deoxyphorbol 13-phenylacetate 20-homovanillate yields a different pattern of response (Szállási *et al.*, 1989b). This compound differs from resiniferatoxin principally by substitution of the orthoester with a monoester and was originally prepared to probe the extent of the structural constraints on the resiniferonol moiety for activity. 12-Deoxyphorbol 13-phenylacetate 20-homovanillate is approximately comparable to resiniferatoxin (or capsaicin) in the eye-wiping assay (Szállási *et al.*, 1989b). It was comparable to capsaicin and four orders of magnitude less potent than resiniferatoxin for inducing oedema in the rat ear (Szállási *et al.*, 1991). Upon systemic administration, 12-deoxyphorbol 13-phenylacetate 20-homovanillate desensitized the rat to neurogenic inflammation with an ED_{50} of approximately 10^{-3} g/kg, whereas it was totally inactive for inducing hypothermia at this concentration (Szállási *et al.*, 1989b). In marked contrast, the ED_{50} of resiniferatoxin for inducing hypothermia was 20-fold lower than the ED_{50} for desensitization of neurogenic inflammation (Szállási and Blumberg, 1989a). Thus, 12-deoxyphorbol 13-phenylacetate 20-homovanillate displays a different pattern of selectivity from either resiniferatoxin or tinyatoxin, being relatively ineffective for induction of hypothermia.

Mezerein 20-homovanillate differs from resiniferatoxin by the presence of a benzoate rather than phenylacetate orthoester, a 6,7-epoxide in place of the 6,7-double bond, and an additional C-5 hydroxyl and a C-12-ester (Figure 2). For protein kinase C, none of these changes has great impact on binding affinity, and the parent moiety mezerein possesses a nanomolar binding affinity. As a vanilloid, in contrast, mezerein 20-homovanillate was virtually inactive (Szállási *et al.*, 1989b).

The mechanistic basis for these differences in pattern of response has not been defined. The binding potencies best correlate with neurogenic inflammation and do not correlate with neurogenic pain (see Section 3.3.1 for discussion). Differences in pharmacokinetics may play a role. Regardless of the mechanism, however, the limited examples available so far document that structural variation leads to striking differences in profile of response.

Additional information on structure activity relations of resiniferonol derivatives, assayed only for ear reddening, is available from the studies of Hecker (Adolf *et al.*, 1982) and of Evans (Schmidt and Evans, 1979). Activity is retained upon acetylation of resiniferatoxin or of tinyatoxin (Schmidt and Evans, 1979), but there is a 100-fold drop in potency upon conversion of the phenolic hydroxyl of tinyatoxin to the butyl ether (Schmidt and Evans, 1979). Conversion of resiniferatoxin to the methyl ether causes a 30-fold drop in potency; similar activity is observed for the 3,4-dimethoxy and the 3,5-dimethoxy derivatives (Adolf *et al.*, 1982). Interestingly, the resiniferatoxin derivative with an unsubstituted phenylacetate ester shows only a 100-fold loss of potency (Adolf *et al.*, 1982); substitution with a long-chain aliphatic ester causes complete loss of potency (Adolf *et al.*, 1982). For the resiniferonol moiety, substitution of the orthophenylacetate by orthoacetate causes a 50-fold loss of potency (Adolf *et al.*, 1982); no activity is observed for the orthotetradecanoate (Adolf *et al.*, 1982).

These results produce the following conclusions. First, the large enhancement of potency provided by the resiniferonol 9,13,14-phenylacetate moiety permits analysis of the contributions of weakly active esters such as 3,4-dimethoxyphenylacetate. With the lesser potency of capsaicin derivatives, compounds such as decanoyl homoveratrylamide had undetectable activity (Szolcsányi and Jancsó-Gábor, 1975). Second, the resiniferonol 9,13,14-orthophenylacetate 20-ester moiety makes a major contribution to potency in the absence of further substitution on the phenyl ring. It therefore defines central elements of the vanilloid pharmacophore.

3.2.4 Time course of action of resiniferatoxin

The three striking features of resiniferatoxin compared to capsaicin are its high potency, its differential spectrum of activity, and its enhanced efficacy to induce desensitization versus pain. Maggi and coworkers have focused attention on a slower onset of action of resiniferatoxin as a possible contributing factor for these differences (Maggi *et al.*, 1990). We have reported that the chemogenic pain response to resiniferatoxin in the rat eye-wiping assay begins 5–10 s after application, compared to an immediate response to capsaicin (Szállási and Blumberg, 1989a). For 12-deoxyphorbol 13-phenylacetate 20-homovanillate the delay was 20–30 s (Szállási *et al.*, 1989b). In the rat vas deferens subjected to repetitive field stimulation, twitch contractions are inhibited by capsaicin or by resiniferatoxin (Maggi *et al.*, 1990). Although resiniferatoxin is 10 000-fold more potent than capsaicin in this system, the time course for development of inhibition was much slower. Capsaicin inhibition appeared at 10–40 s and reached a maximum at 2 min, whereas resiniferatoxin inhibition did not being for at least 60 s and full response only developed at 12 min. In adult rat dorsal root ganglion neurones, likewise, resiniferatoxin caused slower enhancement of membrane conductance than did capsaicin (Winter *et al.*, 1990).

Although the onset of acute response to resiniferatoxin may be slower, the onset of desensitization to neurogenic inflammation by maximally tolerated doses of resiniferatoxin was more rapid than that to capsaicin (Szállási *et al.*, 1989a), with 70% inhibition appearing within 1 h. Further experimentation is required, however, since the greater therapeutic index for resiniferatoxin meant that the animals were not being treated at equi-effective doses. The recovery from desensitization by resiniferatoxin was slower than that for capsaicin (Szállási *et al.*, 1989a). For neurogenic inflammation, complete recovery from capsaicin was achieved by 21 days, whereas recovery from resiniferatoxin was only to 50% of control at this time. For chemogenic pain assayed in the eye-wiping assay, recovery was complete by two weeks for capsaicin and not detectable for resiniferatoxin at 21 days. For hypothermia, recovery from resiniferatoxin desensitization was not detectable at 14 days, upon a challenging dose of 0.6 μg/kg s.c. This challenging dose is two-fold the ED_{50} in control animals. At a challenging dose of 300 μg/kg, no response was detected at 12 h but approximately 50%

recovery was observed for times of 1–14 days. These findings suggest that the recovery from desensitization, at least for hypothermia, reflects two distinct processes with very different rates of recovery. A further conclusion is that, at least for hypothermia, there is a graded sensitivity to resiniferatoxin, and that the neurones more sensitive to acute response are more sensitive to desensitization.

3.3 Specific binding of resiniferatoxin

3.3.1 Specific receptors

The existence of specific receptors mediating vanilloid action seemed highly likely based on the potency and structural specificity of capsaicin and its analogues, as indeed predicted by Szolcsányi (Szolcsányi and Jancsó-Gábor, 1975). The possibility remained, however, that activity represented a high affinity, non-saturable membrane interaction, as exemplified by palytoxin (Moore and Bartolini, 1981). The difficulty in resolving this issue came from the high lipophilicity of capsaicin, its micromolar biological potency, and the low concentration of receptors. These three factors combine to generate extensive non-specific binding, overwhelming specific receptor interactions.

The orders-of-magnitude higher affinity of resiniferatoxin, which represents a much lower concentration of ligand at the K_d, dramatically reduced these technical obstacles. We were thus able to demonstrate and characterize specific, saturable binding of [^{3}H]resiniferatoxin (Szállási *et al.*, 1991; Szállási and Blumberg, 1990a, 1991a, 1992b). The two animal species that we have focused on for characterization of receptors are the rat, because of the wealth of information on biological responses to vanilloids in this species, and the pig, because of its size and availability. Specific binding has been confirmed in other mammalian species, including the cow (Szállási and Blumberg, 1990a) and sheep (Szállási and Blumberg, 1990a).

Details of the binding procedures have been published elsewhere (Szállási and Blumberg, 1992b). Since specific binding is found in the particulate fraction, we have used two types of binding assays to separate bound and free ligand. The centrifugation assay has the advantage that the concentration of [^{3}H]resiniferatoxin remaining free in the supernatant can be measured directly. Since an appreciable amount may be bound to the receptors, be taken up non-specifically by the membranes, or lost onto surfaces or the air–water interface, we currently favour this approach. The filtration assay has the advantage that it is easier to handle large incubation volumes, which may be important when one wishes to maintain a large excess of ligand over receptor while preserving a sufficient total amount of receptor in the assay at a sufficiently low concentration.

The initial assays using rat dorsal root ganglia yielded approximately 50%

specific binding at the K_d for [^{3}H]resiniferatoxin (Szállási and Blumberg, 1990a). Two factors subsequently have substantially improved the ratio of specific to non-specific binding. First is choice of receptor source; dissection of Rexed laminae I and II from pig dorsal horn provides several-fold enrichment over dorsal root ganglia. Second, as detailed below, we have identified α_1-acid glycoprotein as a vanilloid serum binding protein (Szállási *et al.*, 1992). Addition of α_1-acid glycoprotein to the incubation buffer after ligand binding and chilling permits extraction of the unbound ligand from the membranes into the supernatant, thereby reducing non-specific binding, whereas the specifically bound ligand is retained because of the slow off-rate.

[^{3}H]Resiniferatoxin bound to rat dorsal root ganglion membranes with a K_d of 0.27 nM and a B_{max} of 160 fmol/mg (Szállási and Blumberg, 1990a). Binding to pig dorsal root ganglion membranes yielded a weaker K_d of 2.2 nM coupled with a higher B_{max} of 730 fmol/mg (Szállási and Blumberg, 1990a). We were able to assay binding by the central projections of the dorsal root ganglion neurones. The binding to dorsal horn of the pig spinal cord provided a higher affinity, 0.27 nM, than was the case for the cell bodies and a B_{max} of 370 fmol/mg (Szállási and Blumberg, 1991a). The higher affinity of the receptors at the central terminals is consistent with the suggestion that the spinal cord is more sensitive than the periphery to the desensitization effects of capsaicin (Dickenson and Dray, 1991). Although the binding was consistent with a Hill coefficient of 1 and a single component (Szállási and Blumberg, 1990a, 1991a), these conclusions were derived from assays in the absence of α_1-acid glycoprotein. The better ratio of specific to non-specific binding under the latter condition indicates positive cooperativity with a Hill coefficient of approximately 2 (Szállási *et al.*, 1993b).

The crucial distinction between a specific binding protein and a receptor is whether the protein mediates the biological responses. The specific [^{3}H]-resiniferatoxin binding displays the overall species, tissue and pharmacological specificity appropriate for the vanilloid receptor. As described above, specific binding is observed in a variety of mammalian species. It is not found, however, in chickens (Szállási and Blumberg, 1990a) or in alligators (Szállási and Blumberg, 1993). Birds are recognized to be unresponsive to capsaicin (Pierau *et al.*, 1986), and we have shown that alligators are likewise unresponsive (Szállási and Blumberg, 1993b). We had examined the alligators because of an earlier report claiming that crocodiles responded to capsaicin (Kanui *et al.*, 1990), contrary to the general view that only mammals are responsive. Since differences in relative sensitivity of mammalian species have been reported, for example hamsters and rabbits are relatively resistant to capsaicin (Glinsukon *et al.*, 1980), examination of such species should be of future interest.

Both dorsal root ganglia and dorsal horn of the spinal cord, the tissues in which specific binding has been identified, have been clearly shown to be involved in the biological action of vanilloids. Cerebellar membranes were used as a negative control (Szállási and Blumberg, 1990a). As yet, the binding assay has

not sufficed to detect resiniferatoxin binding in tissues with, presumably, a lower receptor density. Tissues examined for receptors on peripheral terminals include cornea, tooth pulp and ureter (unpublished observations). Likewise, binding has not yet been detected in the preoptic area of the brain, the striatum and the substantia nigra (Szállási and Blumberg, 1990a).

Although the species and tissue distribution is consistent with the behaviour predicted for the vanilloid receptor, the strongest evidence for its identity is its structure–activity relations (Szállási *et al.*, 1991; Szállási and Blumberg, 1990a, 1991a). Capsaicin displaced specific resiniferatoxin binding to rat dorsal root ganglia with a K_i of 7 μM (Szállási and Blumberg, 1990a) or 900 nM (Szállási *et al.*, 1991). The value for pig dorsal root ganglia was 14 μM (Szállási and Blumberg, 1990a); for pig dorsal horn it was 1.9 μM (Szállási and Blumberg, 1991a). Capsaicin was thus three to four orders of magnitude less potent than resiniferatoxin, consistent with the relative potencies for neurogenic inflammation and for hypothermia. Tinyatoxin and 12-deoxyphorbol 13-phenylacetate 20-homovanillate inhibited binding with K_i values of 0.24 nM and 600 nM, respectively (Szállási *et al.*, 1991). Their potencies for inducing ear oedema in the rat were approximately those for resiniferatoxin and capsaicin, respectively (Szállási *et al.*, 1991). Capsaicin analogues which inhibited binding included homovanilloylamides with chain lengths of 10 to 16 carbons (Szállási *et al.*, 1991). Interestingly, the homovanilloyl hexadecylamide revealed slow kinetics of equilibration. Pharmacokinetic factors may thus contribute to its relatively low pungency. A similar factor may account for the low irritancy of olvanil (Dickenson *et al.*, 1990).

Compounds which failed to inhibit binding included the following: nonenoyl-homoveratrylamide and homovanilloyl-piperidine (30 μM) (Szállási *et al.*, 1991); phorbol 12,13-dibutyrate (100 μM) and resiniferonol 9,13,14-orthophenylacetate (30 μM), the diterpene moiety of resiniferatoxin lacking the homovanillyl ester (Szállási and Blumberg, 1991a); piperine (100 μM) and zingerone (100 μM) (Szállási and Blumberg, 1991a). The lack of activity of these latter two compounds may reflect their weak biological potency and low relative solubility.

Other potential classes of inhibitors examined either because of structural similarity or physiological activity on the C-fibre pathway are: *o*-chloroacetophenone (100 μM) (Szállási *et al.*, 1991), prostaglandin E_1 (1 mM) (Szállási *et al.*, 1991), prostacyclin (1 mM) (Szállási *et al.*, 1991), ruthenium red (10 μM) (Szállási and Blumberg, 1990a), and adrenalin, noradrenalin, L-dopa and dopamine (all at 100 μM) (Szállási and Blumberg, 1990a).

Initial physical characterization of the vanilloid receptor has been provided by radiation inactivation analysis (Szállási and Blumberg, 1991b). This technique is able to determine a molecular mass for the functional binding entity. The molecular mass was 270 ± 25 kDa, consistent with the size for other ligand-gated cation channels such as the acetylcholine receptor or the benzodiazepine receptor. Photoaffinity labelling with capsaicin analogues had earlier indicated molecular masses of 58 kDa and 42 kDa (Wood *et al.*, 1990). This labelling was evidently

not related to specific capsaicin receptors, since it was similar in rats and chickens, although chickens are unresponsive to capsaicin.

Resiniferatoxin treatment leads to long-term desensitization. Vanilloid receptor levels in adult rats remained unaltered at 6 h (Szállási and Blumberg, 1992a) or 12 h (Szállási and Blumberg, 1990a) after resiniferatoxin treatment, but then declined by 80–90% by 24 h (Szállási and Blumberg, 1992a). Since numbers of dorsal root neurones were similar before and after treatment, loss of neurones did not account for the receptor loss (Szállási and Blumberg, 1992a). Recovery of binding occurred slowly, with 20–30% restoration by 28 days (Szállási and Blumberg, 1992b); over this period the neurogenic inflammatory response recovered to 60% and no recovery of response to chemogenic pain was observed (Szállási and Blumberg, 1992a). Comparison of dose–response curves for desensitization and for loss of vanilloid receptors indicated that desensitization to chemogenic pain occurred with an ED_{50} 30–60-fold lower than for receptor loss; desensitization to neurogenic inflammation occurred with an ED_{50} six-fold lower than that for receptor loss (Szállási and Blumberg, 1992b).

In rats treated as newborns with resiniferatoxin (300 μg/kg) and evaluated at six to eight weeks, specific resiniferatoxin binding was 10–20% of controls (Szállási *et al.*, 1990). Unlike the rats treated as adults, approximately 50% loss of neurones was observed in the rats treated as newborns. Neurogenic inflammation in response to xylene was 12% of that of control animals; no chemogenic pain response was observed.

The biological assays had indicated marked diversity in the patterns of response to different vanilloids. Although the [^{3}H]resiniferatoxin binding detects a single class of receptors, we think it highly likely that receptor subclasses with different structure activity relations may exist. Since chemogenic pain shows very distinct structure activity relations, e.g. resiniferatoxin and capsaicin are almost comparable in potency, we compared [^{3}H]resiniferatoxin binding by Gasserian (trigeminal) and dorsal root ganglia (Szállási and Blumberg, 1990a). Similar absolute potencies and structure activity relations were observed. One plausible explanation is that a second class of receptors is indeed present in the Gasserian ganglia. Since these latter receptors have only a low affinity for resiniferatoxin, however, binding to them is below the limits of detection.

A very different view of resiniferatoxin receptors is provided by the laboratory of Evans (Ryves *et al.*, 1989). They fractionated a lysate of human mononuclear cells on hydroxyapatite; they found a peak of phospholipid-dependent, Ca^{2+} inhibitable histone kinase activity which was activated both by phorbol 12-myristate 13-acetate and by resiniferatoxin. Unfortunately, the significance of this report is difficult to assess, since resiniferatoxin was included in the assay at the concentration of 9 μM. At such concentrations, an effect of resiniferatoxin could well be due to perturbation of the lipid phase in the assay or to weak potency as an activator of a species of protein kinase C. We had reported a K_i of 400 nM for resiniferatoxin binding to a mixture of the alpha, beta and gamma isozymes of protein kinase C (Szállási *et al.*, 1989b).

3.3.2 Binding by α_1-acid glycoprotein in serum

The high potency of resiniferatoxin for its receptors, the exquisite specificity for its binding, and the teleologic argument that evolution is selecting toxin structures to interact with pre-existing target sites (for which the enkephalins and endorphins as endogenous opiates is the classic example) cogently argue for the existence of endogenous vanilloids. Against this probability is the experimental evidence that capsazepine does not have biological effects in the animals in the absence of capsaicin (Dickenson and Dray, 1991). Inhibition of [^{3}H]resiniferatoxin binding to its receptors provides one approach to demonstrating and identifying endogenous vanilloids if they exist.

As part of our screening effort, we observed inhibition of [^{3}H]resiniferatoxin binding by serum (Szállási *et al.*, 1992). Since non-specific as well as specific resiniferatoxin binding was reduced in parallel, the evident mechanism was ligand sequestration rather than a direct interaction at the receptor *per se*. The two major drug binding proteins in serum are serum albumin and α_1-acid glycoprotein (also known as orosomucoid). Direct assay with the individual purified proteins confirmed that α_1-acid glycoprotein bound resiniferatoxin. The dissociation constant was 0.33 ± 0.07 μM with a stoichiometry approaching 1. Capsaicin also bound to α_1-acid glycoprotein. Its affinity was approximately 35-fold weaker, however, with a dissociation constant of 10.5 ± 3.4 μM. Although serum albumin did not bind, some preparations of serum albumin are contaminated with α_1-acid glycoprotein to a variable degree; this contamination is not surprising since serum albumin is in Cohn fraction V and α_1-acid glycoprotein is in Cohn fraction VI.

The finding that resiniferatoxin binds to α_1-acid glycoprotein has three implications. First, it represents a specific binding site distinct from the vanilloid receptor. It can be distinguished from the vanilloid receptor by its low relative affinity and by its structural requirements for binding: binding to α_1-acid glycoprotein is two to four orders of magnitude lower in affinity than that to the specific receptors on dorsal root ganglia and in the spinal cord; resiniferonol 9,13,14-orthophenylacetate, for example, is only an order of magnitude less potent than is resiniferatoxin. Because α_1-acid glycoprotein exists as a cell-bound form on lymphocytes, granulocytes and monocytes as well as in serum, low affinity resiniferatoxin binding would be expected on such cells.

The second implication is that the presence of serum binding will distort quantitative measures of resiniferatoxin and capsaicin potency in cultures with serum and in the intact animal. The expression for this effect is

$$\text{proportion free ligand} = K_d/(K_d + [\text{AGP}]),$$

where [AGP] is the concentration of α_1-acid glycoprotein and K_d is the dissociation constant of the ligand for α_1-acid glycoprotein. For resiniferatoxin the percentage free would be approximately 15% at the reported plasma concentration of α_1-acid glycoprotein of 4 μM. For capsaicin the percentage free

would be 70% at low concentrations and correspondingly greater for concentrations in the micromolar range and above. α_1-Acid glycoprotein is an acute phase protein, and its elevation in response to inflammation has been extensively characterized (Kremer *et al.*, 1988). Inflammatory states may therefore affect responsiveness to resiniferatoxin by modulating the level of the binding protein.

The third implication, as discussed above, is that α_1-acid glycoprotein can be used to reduce markedly non-specific binding in the vanilloid receptor assay.

3.4 Future directions

The most dramatic contribution of resiniferatoxin has been to show that there exists a class of structures with orders of magnitude greater potency for the vanilloid receptor than had been anticipated through medicinal chemical approaches to modification of capsaicin. Although the mechanistic basis remains to be clearly delineated, resiniferatoxin further displays a substantial enhancement in therapeutic index. In light of the current attention on use of capsaicin in human therapy (Carter, 1991; Szállási and Blumberg, 1993), evaluation of resiniferatoxin and its analogues for desensitization of vanilloid pathways is of tremendous interest.

A simmering issue in the field is that of subclasses of responses with different structure activity relations. Szolcsányi has described partial dissociation between pungency and desensitization for vanilloids of the capsaicin class (Szolcsányi and Jancsó-Gábor, 1975). The Sandoz group has characterized differential central and peripheral effects of the capsaicin analogue olvanil (Dickenson *et al.*, 1990). In our limited studies with vanilloid derivatives of the resiniferatoxin class, it was striking that each derivative displayed a different spectrum of relative potencies for different biological endpoints. It is possible that some of these differences reflect pharmacokinetics. We are proceeding on the assumption, however, that vanilloid receptor subclasses exist with distinct structure activity relations. Cloning of the putative receptor subclasses and analysis of their individual structure activity requirements could have revolutionary impact on the development of yet more selective agents.

The year 1992 is the 500th anniversary of the discovery of the New World by Columbus and the introduction of capsaicin to the Old World. Although controversy may exist on whether other Europeans preceded Columbus, our findings unambiguously demonstrate that the vanilloid field in the Old World antedates Columbus by a millenium and a half. Euphorbium, the dried latex of *E. resinifera*, was described in 30 BC as being a useful therapeutic (Kohler, 1883).

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CHAPTER 4

STRUCTURAL REQUIREMENTS FOR CAPSAICIN AGONISTS AND ANTAGONISTS

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4.1 Introduction

Capsaicin is a pungent compound, produced by chilli peppers and related plants of the *Capsicum* family, which acts specifically on a subset of primary afferent sensory neurones to open cation-selective ion channels (Bevan and Szolcsányi, 1990). This agonist effect, which is probably initiated by binding to a receptor–ion channel complex in the cell membrane, initially causes excitation of these neurones, but subsequently causes a period of insensitivity to noxious stimuli. In whole animals these two responses are perceived as an initial algesic response followed by a subsequent analgesic effect. Both the excitatory and analgesic effects are thought to be a consequence of increases in cytosolic Ca^{++} concentration caused by opening of the channel; the former by direct stimulation of transmitter (e.g. substance P) release and the latter probably by inhibition of voltage-gated Ca^{++} channels leading to block of further transmitter release.

Since the sensory neurones which are affected are involved in nociception, capsaicin is a prototype for a novel class of analgesics, unrelated to the existing, heavily exploited classes of analgesic agents, the opioids and the NSAIDs. An

CAPSAICIN IN THE STUDY OF PAIN
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Differential Activation and Desensitization of Sensory Neurons by Resiniferatoxin

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Recently, with use of rat dorsal root ganglion (DRG) neurons we have been able to dissociate the binding affinities of vanilloids from their potencies to induce ^{45}Ca uptake, which suggests the existence of distinct classes of the vanilloid receptor (Acs et al., 1996). In the present study, we have demonstrated that the ultrapotent capsaicin analog resiniferatoxin (RTX) desensitized rat DRG neurons to the subsequent induction of ^{45}Ca uptake by capsaicin and RTX with affinity and cooperativity similar to that found for [^{3}H]RTX binding, contrasting with a ~10-fold weaker potency and lack of cooperativity to induce ^{45}Ca uptake. Likewise, the competitive antagonist capsazepine inhibited RTX-induced desensitization with potency similar to that for inhibition of specific [^{3}H]RTX binding, whereas the potency of capsazepine was ~10-fold higher for inhibiting RTX-induced ^{45}Ca uptake. Finally, the noncompetitive antagonist ruthenium red inhibited both the RTX-induced desensitization and ^{45}Ca uptake but showed ~60-fold selectivity for inhibiting RTX-induced desensitization. The RTX-induced desensitization was not associated with loss of specific [^{3}H]RTX binding, suggesting lack of gross cell toxicity. In contrast to RTX, capsaicin caused desensitization with a potency corresponding to that for ^{45}Ca uptake and did so in a noncooperative manner. Unlike the RTX-induced desensitization, the desensitization by capsaicin was blocked by ruthenium red only at doses that blocked ^{45}Ca uptake and depended on external calcium. Our findings provide further support for the existence of vanilloid receptor subtypes on DRG neurons with distinct pharmacology and distinct patterns of desensitization.

Key words: dorsal root ganglion neurons; capsaicin; resiniferatoxin; desensitization; [^{3}H]RTX binding; ^{45}Ca uptake; capsazepine; ruthenium red; pain; rat

A subpopulation of primary afferent neurons, located in the dorsal root and trigeminal ganglia, can be defined by their selective susceptibility to the effects of capsaicin (Buck and Burks, 1986; Holzer, 1991), the major pungent ingredient of hot peppers of the plant genus *Capsicum*.

Several years ago we found that resiniferatoxin (RTX), a naturally occurring irritant tricyclic diterpene (Hergenhahn et al., 1975) that combines structural features of the phorbol ester tumor promoters and of capsaicin, functions as an ultrapotent capsaicin analog (Szallasi and Blumberg, 1989). Qualitatively, RTX induces a pattern of responses generally similar to those observed for capsaicin (Szallasi and Blumberg, 1989). RTX and capsaicin differ, however, in their relative potencies for different responses (Blumberg et al., 1993).

As observed for capsaicin, after initial excitation, RTX treatment also leads to desensitization to subsequent RTX application; in addition, desensitization by either compound leads to cross-desensitization to the other (Blumberg et al., 1993). It was postulated decades ago that pungency of capsaicin analogs is proportional to the desensitization that follows (Jancso, 1968); however, this does not seem to be the case for RTX. For example, similar concentrations of RTX and capsaicin cause contraction of isolated rat urinary bladder, but RTX shows a 1000-fold higher potency to induce desensitization (Maggi et al., 1990). Moreover, RTX, but not capsaicin, can desensitize the pulmonary J1 receptors of the rat without previous excitation (Szolcsanyi et al., 1990).

[^{3}H]RTX shows specific, saturable binding to membranes of sensory afferent neurons, displaying appropriate tissue, species, and pharmacological specificity to represent the vanilloid receptor (Blumberg et al., 1993; Acs et al., 1994a,b). Specific [^{3}H]RTX binding by the above membrane preparations displays sigmoidal saturation kinetics, indicating apparent positive cooperativity (Acs et al., 1994a,b).

The basis for the differences in the pattern of responses to capsaicin and RTX and the divergence between the stimulatory and desensitizing potencies of vanilloids has remained unresolved. Consistent with the existence of receptor subclasses (Holzer, 1991; Blumberg et al., 1993), different vanilloid analogs show different potencies for receptor binding and for induction of ^{45}Ca uptake in dorsal root ganglion (DRG) neurons when assayed under similar conditions (Acs et al., 1996). Likewise, both RTX and capsaicin bind to DRG neurons in a positive cooperative fashion but induce ^{45}Ca uptake in a noncooperative manner (Acs et al., 1996).

In the present study, we have examined whether we can identify any responses in the DRG neurons linked to the high-affinity RTX receptor as defined by [^{3}H]RTX binding. We conclude that this site mediates desensitization of ^{45}Ca uptake to subsequent vanilloid challenge. We further demonstrate that stimulation of the ^{45}Ca uptake site by capsaicin can alternatively desensitize the cells to subsequent vanilloid challenge. These two pathways of desensitization show different sensitivities to ruthenium red and different dependence on external Ca^{2+}. On the one hand, our findings strengthen the evidence for multiple vanilloid receptors;

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on the other hand, they help rationalize the extensive evidence for the complexity of "desensitization" in response to vanilloids (Holzer, 1991).

MATERIALS AND METHODS

Female Sprague Dawley rats (6–8 weeks old, 150–160 gm body weight) were purchased from NCI-FCRDC (Frederick, MD). Animals were allowed to access food and water *ad libitum* throughout the course of the experiments. Animal protocols were approved by the Animal Care and Use Subcommittee, Division of Basic Sciences, National Cancer Institute. [^{3}H]RTX (37 Ci/mmol) was synthesized by the Chemical Synthesis and Analysis Laboratory, NCI-FCRDC. $^{45}CaCl_2$ (23.55 mCi/mg) was purchased from DuPont NEN (Boston, MA). Nonradioactive RTX and capsazepine were from LC Laboratories (Woburn, MA). Capsaicin was from Sigma (St. Louis, MO). Ruthenium red was purchased from Research Biochemicals International (Natick, MA).

Cell cultures. Rat DRG neuron cultures were prepared as described (Acs et al., 1995, 1996). Animals were decapitated under CO_2 anesthesia. The spinal columns were removed aseptically, and DRGs from all levels were dissected out and collected in ice-cold DMEM (Life Technologies, Gaithersburg, MD) containing 0.5% heat-inactivated fetal bovine serum (Life Technologies), 1 mM sodium pyruvate, and 25 mM HEPES. Ganglia were digested with 0.125% collagenase (Sigma) in DMEM for 90 min at 37°C and then for an additional 90 min in fresh collagenase solution. Ganglia were washed twice with DMEM containing 25 mM HEPES and 1 mM sodium pyruvate and were triturated through a flame-polished Pasteur pipette to form a single cell suspension. The cells were pelleted through a cushion of DMEM containing 15% fatty acid-free bovine serum albumin (BSA) (Sigma) to remove myelin debris. Cells were then washed three times with serum-free DMEM and resuspended in the same medium, and the number of viable cells was determined by the Trypan-blue dye exclusion test. Cells were then plated into MultiScreen-DV 96-well filtration plates (Millipore, Marlborough, MA) at a density of $\sim 5 \times 10^3$ cells/well in 100 μl of serum-free medium.

For desensitization experiments, cells were incubated at 37°C in the presence of RTX or capsaicin for the indicated times before measurement of induction of ^{45}Ca uptake. Alternatively, the cells in suspension were treated with RTX and competing ligands (capsazepine or ruthenium red). After incubation at 37°C for 6 hr, cells were washed three times with serum-free DMEM containing 0.25 mg/ml BSA and then challenged with either capsaicin or RTX to induce ^{45}Ca uptake. The efficacy of the washing procedure was quantitated in the following way. Cells were incubated in the presence of 250 pM [^{3}H]RTX (the concentration of RTX used in most experiments for inducing desensitization) for 6 hr. The radioactivity in the cell suspension was determined before washing and after each washing step by scintillation counting. According to these experiments, ~95% of the added radioactive RTX was removed by this washing procedure from the suspension (see *inset* in Fig. 5). The number of viable cells was then determined. Cells were then plated into Multiscreen-DV 96-well filtration plates at a density of $\sim 5 \times 10^3$ cells/well in 100 μl serum-free medium and used for ^{45}Ca uptake and [^{3}H]RTX binding assays.

Measurement of ^{45}Ca uptake by DRG neurons. Freshly dissociated cells in MultiScreen-DV 96-well filtration plates were incubated in a total volume of 0.25 ml of serum-free DMEM (containing 1.8 mM $CaCl_2$) in the presence of 0.25 mg/ml BSA (included to stabilize the compounds in the aqueous solution), 1 μCi/ml ^{45}Ca, and increasing concentrations of the different compounds for 20 min at 37°C (Acs et al., 1995, 1996). Cells were then washed five times with ice-cold DMEM by filtration using a MultiScreen Vacuum Manifold (Millipore). Filters were dried under a heat lamp and punched out into scintillation vials using MultiScreen disposable punch tips, and the radioactivity was determined by scintillation counting. For each data point in each experiment, eight wells were assayed.

Analysis of ^{45}Ca uptake data. Analysis of the ^{45}Ca uptake experiments was performed as described previously (Acs et al., 1996) by computer fit to the Hill equation (Endrenyi et al., 1975). In the case of experiments performed on RTX or capsaicin-pretreated cells, desensitization was defined as the difference (in dpm/well) between the increase in ^{45}Ca uptake in these and in control cells after challenge by capsaicin or RTX. The decrease in the ^{45}Ca uptake induced by vanilloids was plotted against the pretreatment concentration of RTX, and the data were fitted to the Hill equation. Data from competition experiments in which the effect of the desensitizing compound was antagonized by either a competitive (capsazepine) or a noncompetitive (ruthenium red) antagonist were fitted to the modified Hill equation (Davis et al., 1977). Data were fitted to the equations using the computer program MicroCal Origin 3.5 (MicroCal Software, Northampton, MA). For the statistical analysis of the curve fitting to the experimental data, the χ^2 test of goodness of fit was used.

Measurement of [^{3}H]RTX binding by DRG neurons. For [^{3}H]RTX binding assays, cells were plated into Multiscreen-DV 96-well filtration plates (Acs et al., 1996). Immediately after plating, 150 μl of DMEM containing 0.25 mg/ml BSA, [^{3}H]RTX, and nonradioactive ligands was added to each well containing the 100 μl cell suspension, and the plates were incubated in triplicate for 60 min at 37°C. Plates were then chilled on ice, and 1 mg of α_1-acid glycoprotein (AGP, Sigma) in 50 μl of ice-cold serum-free DMEM was added to each well to reduce nonspecific binding (Szallasi et al., 1992). Cells were then washed four times with DMEM (200 μl/well) containing 0.5 mg/ml AGP by filtration using a MultiScreen Vacuum Manifold (Millipore). Filters were dried under a heat lamp and punched out into scintillation vials using MultiScreen disposable punch tips, and the bound radioactivity was determined by scintillation counting. Binding was expressed as femtomoles/10^3 cells; nonspecific binding was determined in the presence of 1 μM nonradioactive RTX.

RESULTS

The potencies of RTX and capsaicin to stimulate the uptake of calcium into rat DRG neurons were determined in the presence of 1 μCi/ml ^{45}Ca. As expected, both ligands induced a dose-dependent increase in ^{45}Ca uptake by the cells with ED_{50} values of 1.24 ± 0.02 nM for RTX and 316 ± 47 nM for capsaicin (mean ± SEM for four experiments each). Hill coefficients for the dose–response curves were close to unity (1.08 ± 0.07 and 1.02 ± 0.06 in the case of RTX and capsaicin, respectively; mean ± SEM for four experiments) ($p > 0.05$; Student's t test), suggesting a noncooperative mechanism of action. These values agreed well with those determined previously (Acs et al., 1996).

We had reported previously (Acs et al., 1996) that RTX displayed 24-fold greater potency for specific binding to DRG neurons than for induction of ^{45}Ca uptake. We were interested in how the low concentrations of RTX, at which binding was measured, affected the induction of ^{45}Ca uptake on subsequent capsaicin treatment. We first determined the effect of RTX pretreatment for different time intervals on ^{45}Ca uptake after challenge with capsaicin. Preincubation of the neurons with 100 pM (a concentration close to the K_d value of RTX for receptor binding) and 250 pM RTX (a concentration that almost saturates RTX binding sites) (Acs et al., 1996) for 10 min had no effect on the level of ^{45}Ca uptake when the cells were stimulated with 3 μM capsaicin (a dose that by itself induces a maximal stimulation of ^{45}Ca uptake by the cells) (Acs et al., 1995, 1996) (Fig. 1*A*). In contrast, a significant decrease of ^{45}Ca uptake (20.8 ± 1.3 and 33.6 ± 2.1% decrease compared with control uptake, respectively; mean ± SEM for three experiments each) ($p < 0.05$; Student's t test) was observed in both cases after 30 min of preincubation with RTX. A maximal effect of RTX pretreatment (55.7 ± 3.3 and 91.2 ± 1.8% decrease in ^{45}Ca uptake in the case of 100 and 250 pM RTX pretreatment, respectively, compared with control cells; mean ± SEM for three experiments each) was reached in both cases by 5 hr of preincubation. On the basis of the above results, a 6 hr preincubation time was used in the subsequent experiments to induce maximal desensitization to RTX.

To determine the concentration dependence of RTX-induced desensitization, neurons were incubated with different concentrations of RTX for 6 hr. This procedure resulted in a dose-dependent reduction in the level of ^{45}Ca uptake after challenge with 3 μM capsaicin (Fig. 1*B*). Fitting the desensitization curves (see Materials and Methods) to the Hill equation yielded an

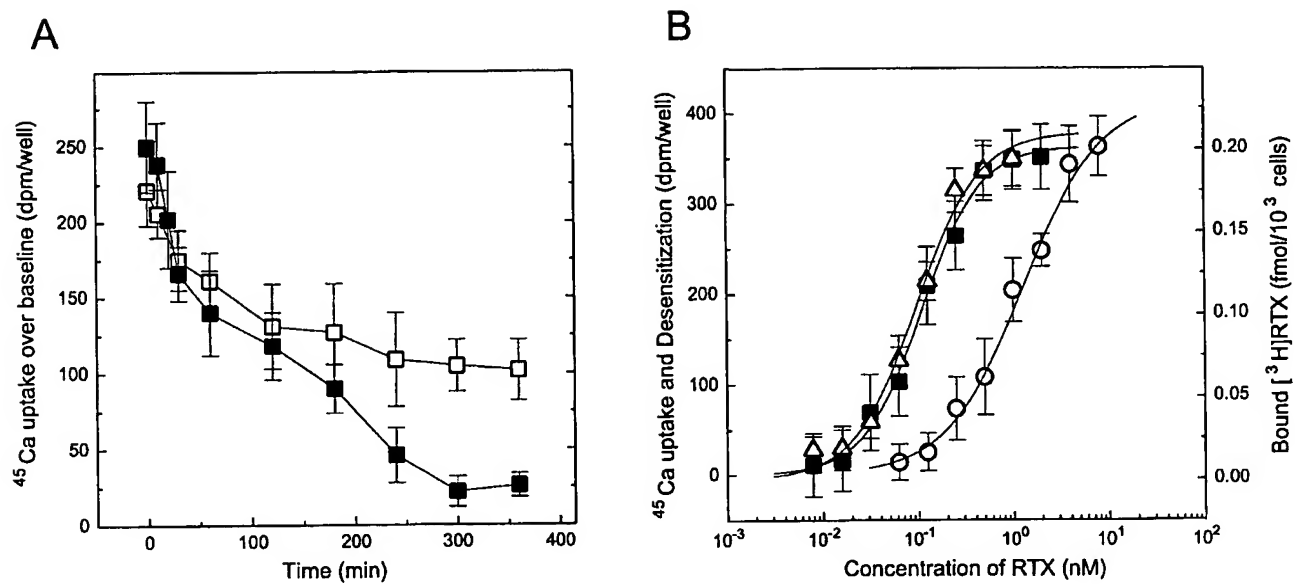

Figure 1. Inhibition of ^{45}Ca uptake in rat DRG neurons after RTX pretreatment. *A*, Time course of the effect of RTX pretreatment on ^{45}Ca uptake. Cells were pretreated with 100 pM (□) and 250 pM (■) resiniferatoxin and then challenged with 3 μM capsaicin. Two additional experiments yielded similar results. *B*, Comparison of dose–response curves for induction of ^{45}Ca uptake, [^{3}H]RTX binding, and desensitization by RTX in rat DRG neurons. Data are expressed as uptake values above baseline for RTX-induced ^{45}Ca uptake (○) and as specifically bound [^{3}H]RTX (△) when binding was determined. Desensitization was defined as the difference (in dpm/well) in ^{45}Ca uptake between pretreated and control cells challenged with capsaicin. In the case of desensitization (■), cells were pretreated with different concentrations of RTX for 6 hr and then challenged with 3 μM capsaicin. Points represent mean values from sets of eight determinations in a single experiment; error bars indicate SEM. The theoretical curves were calculated by fitting the measured values to the Hill equation. In the case of ^{45}Ca uptake and desensitization, three additional experiments in each case yielded similar results. The dose–response curve for [^{3}H]RTX binding is a single experiment, yielding binding parameters similar to those we found previously (Acs et al., 1996).

apparent K_d value for desensitization of 81 ± 5 pM and a Hill coefficient of 1.51 ± 0.11 (mean ± SEM for four experiments), suggesting positive cooperativity of RTX action. The potency of RTX for desensitization thus was ~10-fold higher than that for induction of ^{45}Ca uptake and was similar to its affinity for receptor binding (47 ± 4 pM) (Acs et al., 1996). Moreover, RTX desensitized the cells in a positive cooperative fashion—with a Hill coefficient similar to that determined in receptor binding assays (1.78 ± 0.12) (Acs et al., 1996)—as opposed to its noncooperative action in ^{45}Ca uptake induction assays.

In the above experiments, we desensitized with RTX and challenged with capsaicin. In other experiments we challenged the 250 pM RTX-pretreated cells with as high a dose of RTX as 200 nM. The K_d and Hill coefficient values for desensitization were similar to those after capsaicin challenge (K_d and Hill coefficient values were 91 ± 6 nM and 1.62 ± 0.24, respectively; mean ± SEM for five experiments).

The desensitization of ^{45}Ca uptake was independent of the challenging dose of capsaicin or RTX. Pretreatment of the neurons with 250 pM RTX (a concentration that by itself induced only 11.7 ± 1.5% of maximal ^{45}Ca uptake response; mean ± SEM for four experiments) almost completely (92.1 ± 2.8%; mean ± SEM for four experiments, for challenge by 3 μM capsaicin) abolished the induction of ^{45}Ca uptake by capsaicin up to a concentration of 6.4 μM (data not shown). Likewise, pretreatment of the cells with 250 pM RTX for 6 hr also abolished the ^{45}Ca uptake induced by 200 nM RTX by 90.3 ± 3.1% (mean ± SEM for five experiments) (data not shown). These results suggest that the decrease in ^{45}Ca uptake stimulated by a subsequent challenge could not be attributed to competition at the site coupled to the ^{45}Ca uptake. Further support for this conclusion comes from experiments in which the cells were washed three times after RTX preincubation immediately before the capsaicin challenge (this procedure removed >95% of the 250 pM RTX used for pretreatment) (for details, see Materials and Methods). The washing had no effect on the level of desensitization caused by the pretreatment (data not shown). We conclude that the RTX-induced desensitization does not require the continuous presence of RTX on the receptors.

As shown in Figure 1*B*, [^{3}H]RTX displayed specific binding to rat DRG neurons. At 4 nM [^{3}H]RTX, a concentration sufficient to saturate the receptors, the receptor density in control cells was 0.140 ± 0.002 fmol/10^3 cells. This value agreed well with those determined previously (Acs et al., 1996). Under similar conditions, in cells pretreated with 250 pM RTX for 6 hr and in cells to which 250 pM RTX was added immediately before the binding assay, we determined similar values of specifically bound [^{3}H]RTX of 0.129 ± 0.002 and 0.125 ± 0.001 fmol/10^3 cells, respectively. When we determined the density of RTX binding sites after washing the cells three times after RTX pretreatment, we observed values of specifically bound [^{3}H]RTX of 0.139 ± 0.002 and 0.141 ± 0.008 fmol/10^3 cells, respectively. In neither case did the pretreated cells significantly differ from the corresponding control ($p > 0.05$; Student's t test). We conclude that the observed decrease in ^{45}Ca uptake after RTX pretreatment could not be attributed to the downregulation of the vanilloid receptor as detected by the [^{3}H]RTX binding assay. This observation further demonstrates the lack of toxicity of the 6 hr incubation with 250 pM RTX. Likewise, the number of viable neurons was not changed after RTX treatment, and the baseline

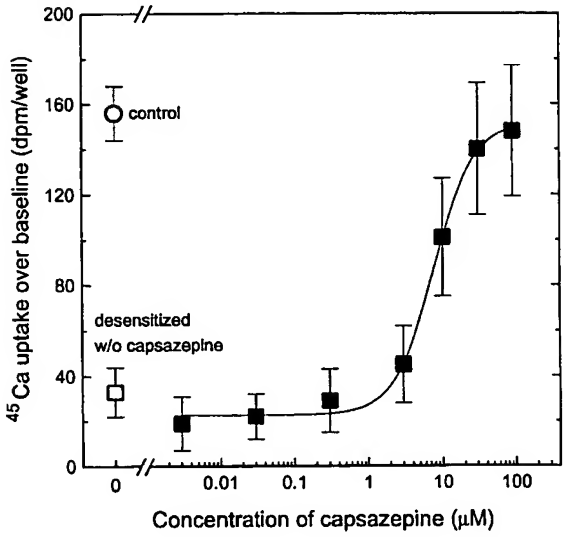

Figure 2. Inhibition of RTX-induced desensitization of rat DRG neurons by capsazepine. Cells were incubated in the presence of 250 pM RTX and different concentrations of capsazepine for 6 hr. Cells were then washed three times with serum-free DMEM containing 0.25 mg/ml BSA to remove the above compounds and were then challenged with 200 nM RTX to induce ^{45}Ca uptake (■). Cells not treated with either RTX or capsazepine (○, control) or treated with only 250 pM RTX for 6 hr (□) are shown as control values. Points represent mean values from sets of eight determinations in a single experiment; error bars indicate SEM. The theoretical curve was calculated by fitting the measured values to the Hill equation. Two additional experiments yielded similar results.

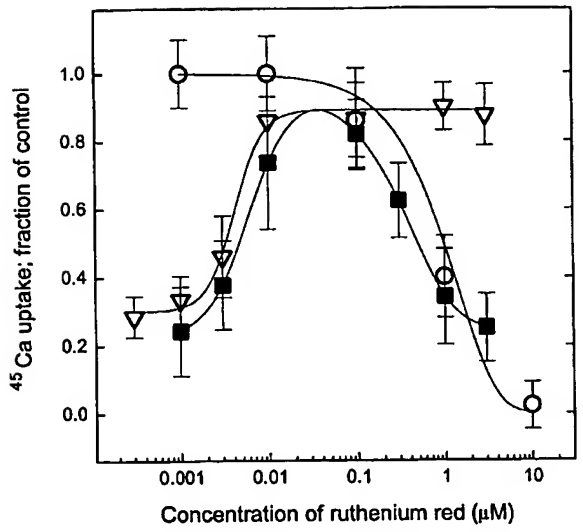

Figure 3. Effect of ruthenium red on RTX-induced ^{45}Ca uptake and on RTX-induced desensitization in rat DRG neurons. Cells were incubated in the presence of 250 pM RTX and different concentrations of ruthenium red for 6 hr and then challenged with 200 nM RTX (■). To evaluate the effect of ruthenium red just on desensitization, after the above incubation cells were washed three times with serum-free DMEM containing 0.25 mg/ml BSA to remove the pretreatment compounds before the cells were challenged to induce ^{45}Ca uptake (▽). To measure the effect of ruthenium red just on ^{45}Ca uptake, varying concentrations of ruthenium red and 200 nM RTX were applied together, and ^{45}Ca uptake was determined (○). Points represent mean values from sets of eight determinations in a single experiment; error bars indicate SEM. The theoretical curve for blocking the RTX-induced ^{45}Ca uptake was calculated by fitting the measured values to the modified Hill equation, whereas data for blocking the RTX-induced desensitization was fitted to the Hill equation. Two additional experiments yielded similar results.

uptake of ^{45}Ca was the same in pretreated and control cells (data not shown).

As observed previously (Acs et al., 1996), the competitive antagonist capsazepine (Bevan et al., 1992; Szallasi et al., 1993) inhibited stimulation of ^{45}Ca uptake by 500 nM capsaicin in a noncooperative fashion. K_i and Hill coefficient values were 291 ± 31 nM and 0.98 ± 0.03, respectively (mean ± SEM for three experiments). Capsazepine likewise inhibited ^{45}Ca uptake into rat DRG neurons induced by 200 nM RTX with similar K_i and Hill coefficient values of 351 ± 39 nM and 1.07 ± 0.08 (mean ± SEM for four experiments) (data not shown).

To determine whether capsazepine also acted as an antagonist of RTX-induced desensitization, we pretreated the cells with 250 pM RTX and different concentrations of capsazepine for 6 hr, washed them three times, and then challenged them with 200 nM RTX. This high challenging concentration of RTX (~200-fold its K_d for induction of ^{45}Ca uptake) was used to assure complete displacement of capsazepine from both the ^{45}Ca uptake site and the specific [^{3}H]RTX binding site. Using the washing protocol (see Materials and Methods), we were able to show that capsazepine inhibited the RTX-induced desensitization in a dose-dependent fashion (Fig. 2), yielding a K_i of 3.66 ± 0.71 μM and a Hill coefficient value of 1.83 ± 0.11 (mean ± SEM for four experiments), suggesting apparent positive cooperativity. These data are in good accord with the K_i and Hill coefficient values of capsazepine for inhibiting specific [^{3}H]RTX binding to DRG neurons (3.16 ± 0.21 μM and 1.72 ± 0.11, respectively) (Acs et al., 1996) and contrast with the respective values of capsazepine for blocking RTX-induced ^{45}Ca uptake (see above). By itself, capsazepine did not cause desensitization.

Similar experiments were performed with the noncompetitive vanilloid receptor antagonist ruthenium red (Amann and Maggi, 1991). When applied together with RTX, ruthenium red blocked the ^{45}Ca uptake into the cells induced by 200 nM RTX in a dose-dependent fashion, with an ED_{50} of 790 ± 40 nM (mean ± SEM for three determinations) (Fig. 3); this value agreed well with those reported previously (Maggi et al., 1988). Furthermore, when administered together with 250 pM RTX during the 6 hr pretreatment, ruthenium red had a biphasic effect on ^{45}Ca uptake induced by a subsequent challenge with 200 nM RTX. At lower concentrations ruthenium red inhibited RTX-induced desensitization, which was reflected by the increased ^{45}Ca uptake into the cells. At higher concentrations, however, ruthenium red inhibited the ^{45}Ca uptake induced by the challenging dose of RTX. When the cells were washed three times after pretreatment with RTX and ruthenium red, the second phase of the above curve was eliminated (no ruthenium red was present in the assay to block ^{45}Ca uptake) (Fig. 3). In these latter experiments ruthenium red inhibited 250 pM RTX-induced desensitization with an ED_{50} of 14 ± 2 nM (mean ± SEM for three determinations), a value markedly different (~60-fold difference in potencies) from that determined for the inhibition of induction of the ^{45}Ca uptake. The ability of ruthenium red to inhibit selectively the RTX-induced desensitization is of great importance. It argues strongly that the desensitization observed in response to RTX does not reflect simply a long-term consequence of limited Ca^{2+} influx occurring at a concentration of ligand below the ED_{50} for stimulation of ^{45}Ca uptake.

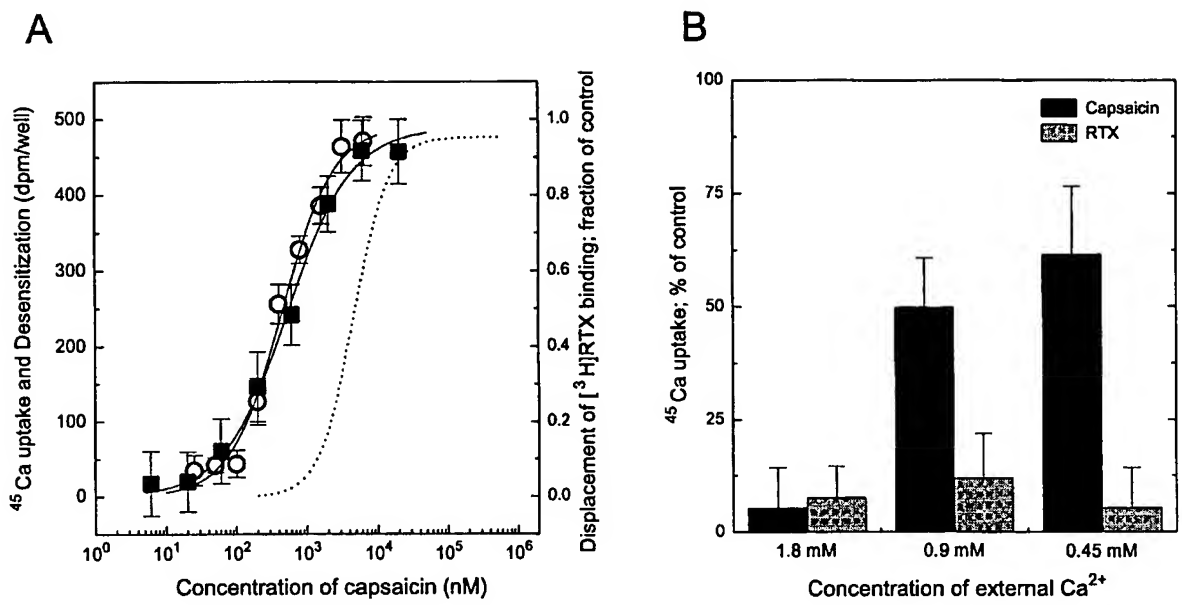

Figure 4. *A*, Comparison of dose–response curves for induction of ^{45}Ca uptake, desensitization, and displacement of [^{3}H]RTX binding by capsaicin in rat DRG neurons. Data are expressed as dpm/well values over baseline for capsaicin-induced ^{45}Ca uptake (○). In the case of desensitization (■), cells were pretreated with different concentrations of capsaicin for 6 hr and then challenged with 3 μM capsaicin. Desensitization was defined as the difference (in dpm/well) in ^{45}Ca uptake between pretreated and control cells challenged with capsaicin. For comparison, the capsaicin dose–response curve for displacement of [^{3}H]RTX binding (*dotted line*), determined previously by us (Acs et al., 1996), was plotted. Points represent mean values from sets of eight determinations in a single experiment; error bars indicate SEM. The theoretical curves were calculated by fitting the measured values to the Hill equation. Two additional experiments in each case yielded similar results. *B*, Extracellular Ca^{2+} dependence of the capsaicin-induced desensitization of rat DRG neurons. Cells were incubated with either 250 pM RTX or 1 μM capsaicin for 4 hr in medium that contained 1.8 mM, 0.9 mM, or 0.45 mM Ca^{2+}. After incubation, cells were washed with serum-free DMEM containing 1.8 mM Ca^{2+} and challenged with 3 μM capsaicin to induce ^{45}Ca uptake. Data were expressed as percentage values of control ^{45}Ca uptake determined on untreated cells in the same medium. The external Ca^{2+} dependence of desensitization is indicated by the return of the ^{45}Ca uptake response. Points represent mean values from sets of eight determinations in a single experiment; error bars indicate SEM. Two additional experiments yielded similar results.

The motivation for the above studies was to determine whether we could identify any responses that were coupled to the vanilloid receptor subtype detected by [^{3}H]RTX binding. We conclude that this receptor subclass is associated with desensitization of subsequent ^{45}Ca uptake in response to vanilloid challenge. These studies do not address the converse issue, whether the vanilloid receptor subtype detected by ^{45}Ca uptake can also induce desensitization. We therefore treated the cultured DRG neurons with capsaicin, which is selective for inducing ^{45}Ca uptake and has weaker affinity for the receptor defined by specific [^{3}H]RTX binding, and we determined the effect of capsaicin pretreatment on the subsequent ^{45}Ca uptake induced by vanilloid challenge. The stimulation of ^{45}Ca uptake was inhibited by capsaicin pretreatment with an ED_{50} of 445 ± 46 nM (Fig. 4*A*). This value compares closely with the ED_{50} for induction of ^{45}Ca uptake by capsaicin, 316 ± 47 nM, and is thus markedly lower than that for inhibition of [^{3}H]RTX binding by capsaicin (K_i of 4.9 μM) (Acs et al., 1996). Furthermore, capsaicin induced desensitization in a noncooperative manner (with a Hill coefficient of 0.89 ± 0.15; mean ± SEM for three experiments), similar to its action on inducing ^{45}Ca uptake (see above) and in contrast to the displacement of [^{3}H]RTX binding (Hill coefficient of 1.81) (Acs et al., 1996).

We next compared the dependence of the desensitization induced by either RTX or capsaicin on the concentration of Ca^{2+} in the medium. Because the DRG neurons show toxicity to prolonged incubation in the absence of extracellular Ca^{2+}, we determined that desensitization in media containing decreased Ca^{2+} concentrations after 4 hr preincubation (Fig. 4*B*). Desensitization by 250 pM RTX was not affected by the change in the concentration of Ca^{2+} in the medium, whereas desensitization by 1 μM capsaicin was reduced in media containing a decreased concentration of Ca^{2+}.

Finally, consistent with the desensitization in response to capsaicin reflecting the induction of ^{45}Ca uptake, we compared the ability of ruthenium red to block the capsaicin and RTX-induced desensitization. We examined concentrations of ruthenium red that were three- to fourfold the ED_{50} for blocking RTX-induced desensitization and ^{45}Ca uptake (60 nM and 2 μM, respectively). Only the latter concentration inhibited the desensitization induced by 1 μM capsaicin; in contrast, the desensitization induced by 250 pM RTX was already inhibited by 60 nM ruthenium red, as expected (Fig. 5). We conclude that capsaicin induces desensitization through a mechanism distinct from that of RTX. The capsaicin-induced desensitization is linked to the enhanced Ca^{2+} influx; the RTX-induced desensitization is mediated by the receptor detected by specific [^{3}H]RTX binding.

DISCUSSION

Our findings cogently argue for the existence of two pharmacologically defined classes of vanilloid receptors, for which we suggest the designation R(TX)-type and C(apsaicin)-type. The distinct pharmacology of these two receptor subclasses had already been demonstrated in our comparison of [^{3}H]RTX binding and ^{45}Ca uptake in intact DRG neurons (Acs et al., 1996). In the present study, we have extended the evidence by showing that a biological response, desensitization in response to RTX, quanti-

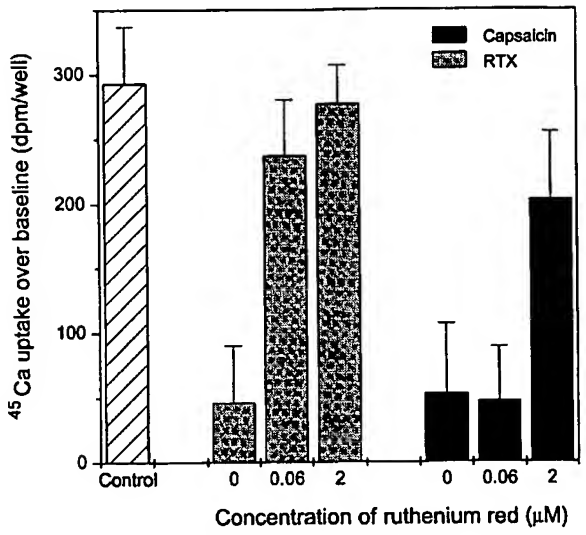

Figure 5. Differential inhibition by ruthenium red of capsaicin and RTX-induced desensitization of rat DRG neurons. Cells were incubated with either 250 pM RTX or 1 μM capsaicin for 6 hr in the presence of 60 nM or 2 μM ruthenium red, washed three times with serum-free DMEM containing 0.25 mg/ml BSA to remove the pretreatment compounds, and then challenged with 3 μM capsaicin to induce ^{45}Ca uptake. The return of the ^{45}Ca uptake response in the presence of ruthenium red represents the inhibition of desensitization. Points represent mean values from sets of eight determinations in a single experiment; error bars indicate SEM. Two additional experiments yielded similar results.

tatively agrees with the pharmacology for the R-type receptor. We further show that ruthenium red, a noncompetitive vanilloid antagonist, shows markedly different affinity for blocking responses through the R- and C-type receptors.

A critical issue was whether the desensitization induced by RTX could simply have been a consequence of a low level of ^{45}Ca uptake induced by RTX occupying a small fraction of the C-type receptors. Three findings argue against this explanation. First, ruthenium red was much more potent for blocking the RTX-induced desensitization compared with ^{45}Ca uptake. Second, the RTX-induced desensitization showed no dependence on external Ca^{2+}, in contrast to the desensitization by capsaicin. Finally, desensitization in response to capsaicin was observed only with an ED_{50} corresponding to that for induction of ^{45}Ca uptake, not at a concentration an order of magnitude lower.

Elegant previous studies have established that capsaicin-induced desensitization is largely dependent on external Ca^{2+} (Santicioli et al., 1987; Cholewinski et al., 1993; Docherty et al., 1996). Our results with capsaicin are consistent with these observations and provide further support for distinct mechanisms of desensitization mediated by the R- and C-type vanilloid receptors. Obviously, the ability to separate responses through these two pathways in DRG neurons will depend on ligands of appropriate selectivity and on their use at appropriate concentrations. At higher concentrations, RTX will also act on the C-type receptors and capsaicin on the R-type. An alternative approach for analysis would be to find systems in which only one receptor subtype is expressed. Elsewhere, we will describe characterization of vanilloid responses in a series of non-neuronal cell lines. In these cells, we observe only C-type receptors and not R-type receptors, and the C-type receptors display characteristics quantitatively similar to those described here (T. Biro, M. Maurer, S. Modarres, N. E. Lewin, C. Brodie, G. Acs, P. Acs, R. Paus, and P. M. Blumberg, unpublished observations).

Our findings help structure and interpret various observations by multiple groups in the vanilloid field. Szallasi and colleagues (1993) (Goso et al., 1993) have reported previously that specific RTX binding in some preparations, e.g., airways and colon, is of low affinity (K_d values for RTX are 250 pM and 3 nM, respectively) and lacks cooperativity; these characteristics might fit with these measurements reflecting interaction at the lower affinity, ^{45}Ca uptake-coupled receptor site. Interpretation had been clouded somewhat because of the difficulties in accurately defining this low-affinity binding and the known variability in the cooperativity depending on the conditions of membrane preparation (Szallasi and Blumberg, 1993). Like us, Walpole et al. (1996) found differences between the structure–activity relations for ^{45}Ca uptake and RTX binding. In their case, however, binding was performed on membrane preparations, and because the general trend was similar for both assays, they concluded that "the potencies in the ^{45}Ca uptake assay are generally about 10-fold lower than binding potencies, presumably due to other processes, e.g., uptake into mitochondria being necessary for detection in this assay." In fact, the differences in structure–activity relations are impressive. If one compares the relative potency for binding versus ^{45}Ca uptake, the ratio (K_i/ED_{50}) is 0.04 for RTX compared with 15 for capsaicin; RTX thus permits a 375-fold better selectivity than does capsaicin for the high-affinity RTX binding site compared with the vanilloid receptor mediating ^{45}Ca uptake.

In biological systems, abundant evidence supports distinct structure–activity relations for different biological responses for vanilloids (Holzer, 1991). Such differences are evident not only in comparisons between vanilloids of the RTX and capsaicin classes, but also within each class. Thus, the vanilloid analog olvanil differentially induces desensitization compared with capsaicin (Dickenson et al., 1990; Dray et al., 1990). The dissection of patterns of biological response can be explained most readily by receptor subtypes, although it is also clear that differences in pharmacokinetics can complicate interpretation (Maggi et al., 1990).

Cellular studies of others likewise argue for vanilloid receptor heterogeneity. Liu and Simon (1994), for example, characterized currents induced in single sensory neurons in response to capsaicin and RTX. At least one subset of currents was inducible by capsaicin but not RTX (at the concentration examined), and furthermore, significant differences were found in the desensitization patterns of the different vanilloid-sensitive currents (Liu and Simon, 1996).

The findings reported here have important implications. First, the evidence that vanilloids induce the activation of a nonvoltage-dependent, relatively nonselective cation channel in intact cells (Wood et al., 1988; Winter et al., 1990) and in single-channel patch-clamp preparations (Oh et al., 1996) has strongly supported the argument that the vanilloid receptor either is, or is closely associated with, this ion channel. Our findings suggest that a subset of vanilloid receptors, namely those corresponding to the RTX selective subclass, have a different mechanism. Although further studies will be required to define the mechanism for the R-type vanilloid receptor, the potent inhibition of desensitization by ruthenium red argues for the involvement of calcium, consistent with activation of, e.g., the phosphoinositide pathway. In fact, the stimulation of the phosphoinositide pathway in DRG neurons after vanilloid treatment has already been described (Harvey et al., 1995).

Elegant studies by Walpole and coworkers (1996) have helped define the structural constraints for activity of vanilloids of the capsaicin class and, to a lesser degree, those of the RTX class. Because these analyses have used ^{45}Ca uptake as the measure of activity, it is clear that separate evaluation of structure–activity relations at the R-type vanilloid receptor will be required. Because this latter receptor is coupled to desensitization without ^{45}Ca uptake, derivatives optimized for selectivity to this site may be of particular potential therapeutic interest. Conversely, vanilloid antagonists selective for the ^{45}Ca uptake site should permit enhanced selectivity when used in combination with a vanilloid agonist selective for the R-type receptor. Indeed, on the basis of our limited knowledge of structure–activity relations for the RTX-selective vanilloid receptor, we should be able to enhance the selectivity of RTX by a factor of 10 by coapplication with capsazepine.

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Exhibit C

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MINIREVIEW

RESINIFERATOXIN AND ITS ANALOGS PROVIDE NOVEL INSIGHTS INTO THE PHARMACOLOGY OF THE VANILLOID (CAPSAICIN) RECEPTOR

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Summary

Capsaicin, the pungent constituent of chili peppers, represents the paradigm for the capsaicinoids or vanilloids, a family of compounds shown to stimulate and then desensitize specific subpopulations of sensory receptors, including C-polymodal nociceptors, A-delta mechanoheat nociceptors and warm receptors of the skin, as well as enteroceptors of thin afferent fibers. An exciting recent advance in the field has been the finding that resiniferatoxin (RTX), a naturally occurring diterpene containing a homovanillic acid ester, a key structural motif of capsaicin, functions as an ultrapotent capsaicin analog. For most of the responses characteristic of capsaicin, RTX is 100-10,000 fold more potent. Structure/activity analysis indicates, however, that RTX and related homovanillyl-diterpene esters display distinct spectra of activity. Specific [^{3}H]RTX binding provides the first direct proof for the existence of vanilloid receptors. We expect that the RTX class of vanilloids will promote rapid progress in understanding of vanilloid structure/activity requirements and mechanism.

I. INTRODUCTION

The hot pepper, with an estimated annual consumption of 125 million pounds in the U.S., represents an aspect of pharmacology intimately familiar to many people. The pungent principle in the hot pepper was identified as capsaicin (Figure 1), and the physiological action of capsaicin has been studied extensively. Following the pioneering work of the late N. Jancso in the late sixties, which demonstrated that sensory nerve endings subserving pain and neurogenic inflammation can be stimulated and then desensitized by capsaicin application (1,2), a growing number of studies have focused on the neuropharmacology of capsaicin analogs, referred to as sensorotoxins, and their clinical relevance (see major reviews:3-7). Our emerging understanding of the mechanism of

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action of capsaicin suggests that capsaicin binds to a putative receptor, thought to be a ligand-gated non-specific cation channel (8), provoking a tetrodotoxin-resistant membrane depolarization and release of inflammatory neuropeptides (5). Capsaicin-induced desensitization and neurotoxicity are thought to be due to a combination of intracellular calcium accumulation and the osmotic stress associated with cation influx (8,9), and to block of retrograde nerve growth factor transport (reviewed in 3); there are thus parallels between the capsaicin-type sensorotoxins and the broader family of excitotoxins, such as excitatory amino acids (10). Further complexity is suggested by whole animal studies, which indicate that capsaicin-induced excitation, acute and chronic desensitization, and neurotoxicity may be partially dissociable (7,11). The major factor which limits the use of capsaicin as a specific neuropharmacological probe is its poor potency; at its in vivo (10^{-2}-10^{-1} g/kg) or in vitro (10^{-6} M) effective dose non-specific effects may occur. Capsaicin thus inhibits a variety of enzymatic activities (12), can cause liver necrosis (13), or can inhibit nerve conductance in species such as frogs (14), snails (15) or chicken (16) which lack the physiological responses typical of mammals (1). The structure-activity relations of capsaicinoids have been explored in depth, but compounds of appreciably greater potency than capsaicin were not identified (17,18).

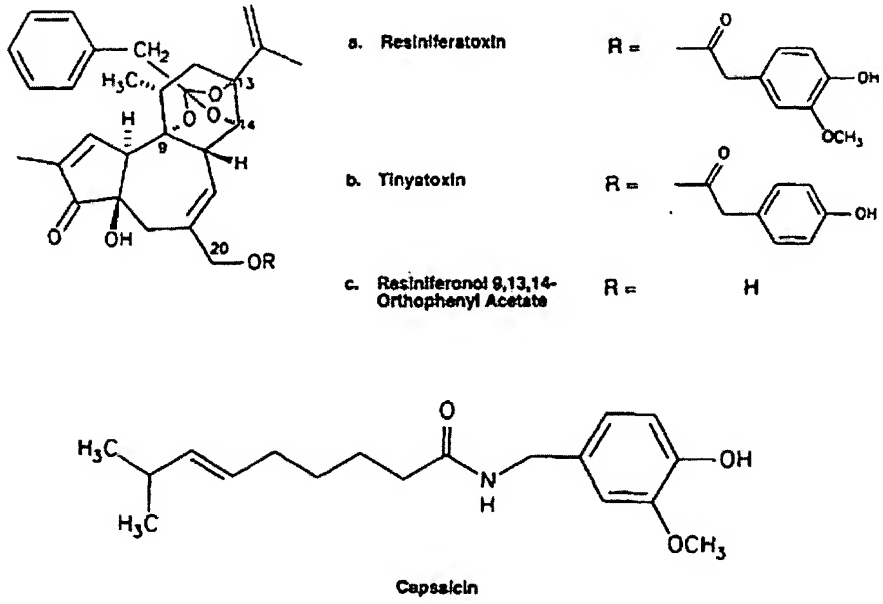

Figure 1.
Vanilloid structures

II. RESINIFERATOXIN, A NEW PROBE FOR CAPSAICIN-SENSITIVE NEURAL PATHWAYS

A. Resiniferatoxin is not just another inflammatory diterpene

Inflammatory diterpenes of the phorbol, ingenol, and resiniferonol series have long been of interest as tumor promoters (19) and as activators of protein kinase C (20). Resiniferatoxin, a diterpene naturally occurring in the latex of Euphorbia resinifera, E. poissonii, and E. unispina, was isolated in 1975 by Hecker on the basis of extraordinary irritant activity in the mouse ear erythema assay (21), in which it exhibited three orders of magnitude greater potency than the most potent of the typical phorbol esters, phorbol 12-myristate 13-acetate (PMA or TPA) (22). Characterization of RTX strongly argued that, despite the structural similarity between RTX and phorbol esters, RTX was not tumor promoting and its primary target was distinct from the phorbol ester receptor, protein kinase C (23-27). These biological findings were consistent with the structure-activity relations for protein kinase C. The diterpene esters which activate protein kinase C have a free OH group at C-20, and modification of this group leads to partial or complete loss of inflammatory potency (28). In contrast, RTX is esterified with homovanillic acid at C-20 (Figure 1), and this substituent is essential for its unusual irritant activity (25,29). A homovanillyl group is likewise a critical structural feature of capsaicin (Figure 1) (2,30). Based on this structural homology between these two classes of irritant compounds, we postulated that RTX and capsaicin might share a common mechanism of action.

B. Resiniferatoxin and capsaicin share a common mechanism but their spectra of action are not identical

B/1. Physiological evidence

Capsaicin causes diverse physiological effects, of which pain, neurogenic inflammation, and hypothermia are prominent examples (1,2). We found RTX to be 3-4 orders of magnitude more potent than capsaicin for induction of acute hypothermia both in the rat and in the mouse (31,32), and for neurogenic inflammation in the rat (32). These acute responses were followed by loss of thermoregulation, by desensitization to neurogenic inflammation, and by chemical and thermal analgesia, with cross-tolerance between RTX and capsaicin (32). RTX was only comparable in potency to capsaicin, however, in the assay for induction of acute pain (protective eye-wiping movements in the rat in response to intraocular instillation of the agents); conversely, the desensitization of this response by means of s.c. administration required less RTX than did desensitization of the other responses (32). The RTX analog tinyatoxin likewise showed low potency in the acute pain assay: Tinyatoxin, which is only 3-5 fold less potent than RTX in its inflammatory (25) and hypothermic effects (manuscript in preparation), was only marginally active in provoking eye-wipings after instillation into the eye. Differential relative potency for different endpoints was also observed for 12-deoxyphorbol 13-phenylacetate 20-homovanillate (Figure 1). 12-deoxyphorbol 13-phenylacetate 20-homovanillate was similar to RTX in the eye-wiping assay. However, whereas RTX was 25-fold more potent for induction of hypothermia than for desensitization of

neurogenic inflammation, 12-deoxyphorbol 13-phenylacetate 20-homovanillate had no effect on thermoregulation at its ED_{50} for desensitization of neurogenic inflammation (33). Some of these differences may reflect pharmacokinetics. Based on studies in rat urinary tract, Maggi and coworkers have suggested that the penetration rate of RTX in tissues is much slower than that of capsaicin, and that this factor can strongly influence the relative observed potencies (34). A slower onset of eye-wiping in response to RTX as compared to capsaicin had similarly been noted (32). Independent of the explanation, it is certain that the RTX class of vanilloids can yield large relative differences in potency compared to capsaicin for different endpoints.

Following treatment of rats with a maximally tolerated single dose of RTX or capsaicin, different responses recovered at different rates following desensitization. All endpoints recovered later after desensitization with RTX, however, than with capsaicin (35). Of particular interest was the persistent desensitization to chemically-induced pain with retention of the ability of the rats to thermoregulate at elevated temperatures (35).

Among the capsaicin-sensitive neural pathways, the pulmonary chemosensitive receptors that trigger the pulmonary chemoreflex (J-receptors) are of particular interest due to their postulated role in hyperreactive airways (36). It is intriguing that RTX, unlike capsaicin, when given i.v. to Wistar rats, failed to elicit the full triple response of bradycardia, hypotension, and apnea. However, it desensitized the J-receptors to capsaicin as well as to phenyldiguanide, a commonly used agent to stimulate these receptors (submitted). A differential sensitivity of pulmonary enteroceptors to RTX and capsaicin had previously been suggested in the comparisons of systemic desensitization (32,35). Whereas capsaicin caused respiratory distress in rats at its ED_{50} for desensitization against neurogenic inflammation, RTX only did so at a dose 2 orders of magnitude above its ED_{50} (32,35).

In addition to the detailed comparison of RTX and capsain actions in the rat, potencies of the two agents were also determined in two standard analgesia assays in the mouse. Campbell and coworkers (37) found RTX to be 1000-fold more active than capsaicin in increasing the tail-flick latency (thermal analgesia), and 5000-fold more potent for the reduction of acetic acid-induced writhings (chemical analgesia).

B/2. Neuromorphological and <u>in vitro</u> electrophysiological evidences

The common mechanism of action for RTX and capsaicin suggested by the physiological studies was further supported by comparison of the neuromorphological effects. RTX, like capsaicin, acts on a particular group of sensory (dorsal root ganglia and Gasserian ganglia) neurons. Swollen mitochondria can be seen in C-type neurons without any alteration in A-type neurons, non-neural elements, or neurons of the capsaicin-insensitive superior cervical ganglion (35). The ultrastructural changes were induced by RTX at a dose 3 orders of magnitude lower than that required for capsaicin (35). The greater effectiveness of RTX compared to capsaicin was also reflected in the induction of calcium staining in DRG neurons (35). In addition, RTX, similarly to capsaicin, depleted SP- and CGRP-like immunoreactivity from both the perikarya and the central

TABLE I.
Absolute and Relative Potencies of Resiniferatoxin and Capsaicin

A., In vivo experiments

A/1. Experiments in the rat

	ED_{50} RTX	CAPSAICIN	RELATIVE POTENCY
hypothermia [32]	3×10^{-7} g/kg	2×10^{-3} g/kg	7000
provoking neurogenic inflammation[1] [32]	3×10^{-5} g/paw	3×10^{-2} g/paw	1000
blocking neurogenic inflammation[2] [32]	5×10^{-6} g/kg	10^{-1} g/kg	20,000
pungency[3] [32]	3×10^{-5} g/ml	3×10^{-4} g/ml	10

A/2. Experiments in the mouse

hypothermia [31]	3×10^{-6} g/kg	7×10^{-3} g/kg	2000
increase in tail-flick latency [37]	2×10^{-6} g/kg	2×10^{-3} g/kg	1000
reducing acetic acid-induced writhings [37]	3×10^{-7} g/kg	2×10^{-3} g/kg	6000
blocking yeast-induced inflammation [37]	3×10^{-6} g/kg	6×10^{-3} g/kg	2000

A/3. Experiments in other species

bradycardia in the cat[4]	1×10^{-7} g/kg	6×10^{-6} g/kg	60
depressor reflex in rabbit ear [34]	N.D.[5]	N.D.	3

[1] neurogenic inflammatory response determined by measuring Evan's blue extravasation on rat paw
[2] desensitization against neurogenic inflammation determined by measuring xylene-induced Evan's blue extravasation on rat paw
[3] pungency determined by counting eye-wipings after intraocular instillation
[4] Szolcsanyi et al., submitted
[5] N.D. = not determined

B., In vitro experiments

	EC_{50} RTX	CAPSAICIN	RELATIVE POTENCY
depolarization of ventral root [45]	N.D.	N.D.	100
^{45}Ca uptake in cultured neurons [38]	2 nM	200 nM	100
^{86}Rb efflux from cultured neurons [38,39]	1 nM	60 nM	60
cGMP induction in cultured neurons [38,40]	1 nM	660 nM	660
twitch inhibition in rat vas deferens[6] [34]	0.01 nM	100 nM	10,000
blocking vas deferens C fibers[6] [34]	0.1 nM	100 nM	1000
contractions of rat urinary bladder [34]	100 nM	100 nM	1
desensitization of rat urinary bladder [34]	0.1 nM	100 nM	1000
SP/CGRP-LI release[7] [34]	N.D.	N.D.	100

[5] twitch contractions was induced by electric field stimulation
[6] vas deferens C fibers was stimulated by capsaicin application
[7] release of SP (substance P)- and CGRP (calcitonin gene-related peptide)-like immunoreactivity from superfused rat dorsal horn slices

endings (dorsal horn) of the C-type DRG neurons (submitted). The relative ability of RTX to release these neuropeptides from superfused slices of the dorsal half of the spinal cord was 100-fold greater as compared to capsaicin (34). Very recently, Winter and coworkers (38) have shown in a combination of electrophysiological and ion flux experiments that RTX and capsaicin induced similar membrane ionic responses in cultured DRG neurons.

The striking differences in absolute potencies between RTX and capsaicin for different in vivo and in vitro responses cannot be explained only by pharmacokinetics (see Table I). In cultured DRG neurons, where problems of absorption should be minimized, RTX induced ^{45}Ca uptake, ^{86}Rb efflux, and cGMP elevation with similar EC_{50}'s of 1-2 nM; capsaicin, in contrast, provoked the same responses with EC_{50}'s of 200 nM, 60 nM, and 660 nM, respectively (38,39,40). Conversely, whilst capsaicin stimulated contractions of rat urinary bladder and blocked the response of vas deferens C-fibers in response to stimulation by electric field or capsaicin with similar potency (approximately 100 nM), RTX yielded EC_{50}'s of 100 nM, 0.01 nM, and 0.1 nM, respectively (34). The most likely interpretation of these contrasting results is a combination of pharmacokinetics and receptor heterogeneity.

III. DISCOVERY OF THE POSTULATED VANILLOID RECEPTOR

The selectivity of capsaicin action and the fairly strict structural requirements for capsaicin-like activity suggested that capsaicin interacts at a specific receptor to produce its effects (2,9,18,30). James and coworkers (41) furnished additional indirect evidence for the existence of capsaicin binding sites by using capsaicin-like photoaffinity probes, which caused irreversible inhibition of response. Whilst the high lipophilicity and the poor potency of capsaicin prevented the use of radiolabeled dihydrocapsaicin to identify capsaicin receptors (41), the use of tritiated-RTX has been proven successful for this purpose (42). [RTX is also highly lipophilic; it might account for the high level of non-specific binding, the major weakness of the present binding assay].

[^{3}H]RTX displayed specific, saturable binding to membranes obtained from sensory (DRG and Gasser) ganglia of rats (42). Computer analysis of the data supported a one site model with dissociation constants (K_d values) of 0.27 and 0.13 nM for DRG and Gasser ganglia, respectively. Capsaicin inhibited specific [^{3}H]RTX binding to DRG membranes with a K_i of 7 uM (42); this difference in potency agrees well with the relative in vivo potencies for neurogenic inflammation and thermoregulation (31,32). Chicken DRG membranes did not show specific RTX binding (42), in agreement with chicken DRG neurons being unaffected by RTX (38).

As yet, the binding data have not yielded evidence for receptor subclasses. The problem may be that the remaining non-specific binding obscures minor binding components.

Although the biochemical nature of the vanilloid receptor remains unknown, the binding assay is providing insight into the

mechanisms of acute desensitization in adults and neurotoxicity in newborns. RTX binding was similar for DRG preparations from control rats and from animals 12 hr after in vivo RTX desensitization (42); the acute desensitization thus does not represent a direct effect of receptor loss. RTX, like capsaicin, caused degeneration of a specific subpopulation of sensory neurons when given to newborns (submitted). Similarly, Bevan and coworkers (38) found that prolonged exposure to capsaicin or RTX damaged a specific subpopulation of cultured DRG neurons. Alternative mechanisms for capsaicin-induced neurotoxicity in the neonate are a higher receptor density in the neonate or an inability of immature neurons to regulate against capsaicin-induced intracellular changes. Limited experiments revealed that neither the vanilloid receptor density nor its affinity in newborns was very different from that of adult DRG neurons (unpublished observations).

IV. PHARMACOLOGY OF THE VANILLOID RECEPTOR

Specific binding of [^{3}H]RTX provides an opportunity for pharmacological characterization of the vanilloid receptor. It is clear that the vanilloid receptor is distinct from protein kinase C, the receptor for typical phorbol esters. Specific RTX binding was not inhibited by phorbol 12,13-dibutyrate, the typical ligand used for analysis of binding to protein kinase C (42). Conversely, RTX inhibited specific [^{3}H]PDBu binding to protein kinase C in the rat DRG preparations only at uM concentrations (42). Bryostatin 1 binds to protein kinase C with high affinity (43). However, bryostatin 26-homovanillate did not inhibit RTX binding to the vanilloid receptor, indicating that protein kinase C and the vanilloid receptor recognize different aspects of the diterpene moiety (unpublished observation).

Capsaicin structural analogs (dihydrocapsaicin, nonanoyl-vanillylamide, piperine and zingerone) showed inhibition of specific [^{3}H]RTX binding in accord with their in vivo potencies (Table II). Structure-activity analysis of RTX analogs indicated that both the homovanillyl substituent and the diterpene moiety play an important role in the high binding affinity of RTX. If the homovanillyl substituent was missing (resiniferonol 9,13,14-orthophenylacetate, the 20-deacylated parent structure of RTX) the affinity for the vanilloid receptor was lost (42). Likewise, this compound was found to be totally inactive for the biological responses characteristic of RTX (33). Replacing the homovanillyl (4-hydroxy-3-methoxyphenyl) substituent with a 4-hydroxyphenyl group, such as found in tinyatoxin, resulted in a 3-fold decrease in the K_i value, in excellent agreement with the relative in vivo potencies of tinyatoxin as compared to RTX (manuscript in preparation). Modification of the diterpene moiety (e.g. removal of the orthoester group, such as found in the 12-deoxyphorbol 13-phenylacetate 20-homovanillate; dPP-HV) also dramatically reduced the affinity, in keeping with dPP-HV being 2-4 orders of magnitude less active in the in vivo assays of inflammation and thermoregulation (33). The parent derivative 12-deoxyphorbol 13-phenylacetate, like the resiniferonol 9,13,14-orthophenylacetate, was inactive in competing for RTX binding sites.

TABLE II.
Comparison of Receptor Binding Affinities of Resiniferatoxin, Capsaicin and Structural Analogs with their Relative *in vivo* Potencies Compared to Capsaicin

	K_i (nM)	Potency Relative to Capsaicin: Hypothermia in rats	Hypothermia in mice	Pungency[1]
Resiniferatoxin	0.1	7000 [32]	2000 [31]	10 [32]
Tinyatoxin	0.3	-	700[2]	-
Capsaicin	7000	1	1	1
Dihydrocapsaicin	3000	1 [18]	-	-
Nonanoyl-vanillylamide	7000	0.1 [17]	-	0.7 [30]
Piperine	> 30,000	0.3 [17]	-	0.015 [30]
Zingerone	> 30,000	-	-	0.001 [30]

[1] pungency determined by counting eye-wipings after intraocular instillation
[2] unpublished observations

The results with RTX strongly support the concept that pharmacological selectivity of vanilloids can be achieved through structural modification. This concept already has received considerable support from analysis of capsaicin congeners (18,30). Additional implications are that RTX may have a more favorable ratio of desensitizing to nociceptive activity, as evidenced in both the rat eye-wiping (32) and rat bladder contraction assays (34), and that the eye-wiping assay may not give a representative measure of vanilloid activity.

Inorganic calcium channel blockers (nickel, cadmium) and Ruthenium Red, known to block RTX/capsaicin actions (39), or bile salts (sodium deoxycholate), shown to facilitate capsaicin effects (44), failed to inhibit [^{3}H]RTX binding at biologically effective concentrations, indicating that should any interaction between these compounds and RTX/capsaicin occur it does not involve the RTX binding site *per se*.

V. FUTURE PERSPECTIVES

Capsaicin is widely used as a neuropharmacological probe to study sensory neuron function. Desensitization of this pathway has been suggested to be a potential therapeutic approach in the relief of neuralgic and post-surgical pain, and commercial capsaicin creams are already in use for this purpose in human therapy. A mechanistic understanding of the pharmacology of the vanilloid receptor may promote rapid progress in experimental drug design. RTX represents a powerful new tool for the investigation of sensory neurons, and the RTX class of vanilloids may further allow dissection of possible subclasses of capsaicin responses. [^{3}H]RTX binding provides a promising opportunity for the analysis of the pharmacology of the vanilloid receptor, for the isolation of the receptor, or for the detection of putative endogenous analogs.

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Mechanisms and Therapeutic Potential of Vanilloids (Capsaicin-like Molecules)

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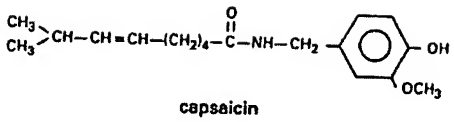
I. Introduction

Capsaicin is most commonly recognized as the active ingredient in hot peppers of the plant genus *Capsicum*. People who fancy "hot," spicy food know the predominant pharmacological actions of capsaicin from experience: on contact with skin or mucous membranes capsaicin causes *irritation* (a burning sensation coupled with wheal-and-flare reaction); the intensity of the irritation gradually diminishes during repeated contacts, and ultimately the affected area becomes insensitive to a variety of noxious stimuli (*desensitization*). Oral consumption of capsaicin leads to profuse

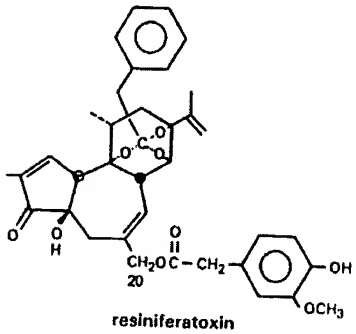
perspiration, termed *gustatory sweating* (*effect on thermoregulation*), and may trigger vegetative reflex responses such as cough and salivation. The capsaicin-induced heat loss may provide a scientific basis for the popularity of "hot" foods in hot climates.

Capsaicin and its structural analogs (capsaicinoids) are ingredients in a variety of traditional remedies used in pain relief, such as oil of clove used to mitigate toothache (Lembeck, 1987). Capsaicin was introduced into experimental pharmacology in the 1960s as a remarkably selective modulator of polymodal nociceptors (Janeso, 1960, 1968); clinical trials to evaluate the efficacy of topical capsaicin to relieve neuropathic pain started in the early 1980s (Bernstein, 1987; Maggi and Meli, 1988); and capsaicin creams in an emollient base (Zostrix, 0.025% capsaicin; Axsain, 0.075% capsaicin) are already commercially available. Nonetheless, clinicians are still reluctant to use capsaicin for several reasons. (1) It has a reputation of being neurotoxic. (2) In contrast to the well-documented beneficial effects of systemic capsaicin in animal experimentation, the efficacy of topical capsaicin administration in clinical trials is so far inconclusive. (3) Because of the marked species differences in capsaicin actions it is difficult to extrapolate to human beings or to predict adverse reactions, which is a serious concern as (4) there has been no known capsaicin antidote.

Until 1989, capsaicin had represented the most active member of a family of naturally occurring (such as piperine and zingerone) and synthetic compounds, collectively referred to as *capsaicinoids* (Szolcsanyi, 1982). Capsaicin research has been given impetus by our recent discovery that a class of naturally occurring, phorbol-related compounds, the paradigm of which is resiniferatoxin (RTX) (Hergenhahn *et al.*, 1975) (see structure in Fig. 1), functions as ultrapotent capsaicin analogs (Szallasi and Blumberg, 1989, 1990b). It has turned out that RTX has a peculiar spectrum of actions. For example, whereas for most of the responses characteristic of capsaicin, RTX is 100- to 10,000-fold more potent (Szallasi and Blumberg, 1990b) (Table 1), it is only equal in potency to capsaicin in a standard assay (protective eye wipings in the rat) used to assess acute, chemically induced pain (Szallasi and Blumberg, 1989). Importantly, we have also shown that (1) RTX not only is an ultrapotent capsaicin analog but also has unique effects, such as the desensitization without excitation of pulmonary J receptors (Szolcsanyi *et al.*, 1990), and (2) the esterification of phorbol-related diterpenes with homovanillic acid can yield further capsaicin analogs with distinct spectra of activity (Szallasi *et al.*, 1989b). Specific [^{3}H]RTX binding has provided the first direct proof for the existence of capsaicin receptors (Szallasi and Blumberg, 1990a, 1991a), and now represents a biochemical means for exploring their pharmacology (Szallasi and Blumberg, 1990b, 1992a).



capsaicin



resiniferatoxin

Fig. 1 Structures of capsaicin and resiniferatoxin.

As RTX structural analogs and capsaicinoids differ dramatically in the rest of the molecule but share a homovanillyl group as a structural motif essential for biological activity (Fig. 1), these bioactive compounds now appear to be best termed *vanilloids*.

We anticipate that the preceding developments along with the synthesis of a functional capsaicin antagonist (Bevan *et al.*, 1991) and with our emerging understanding of the role that capsaicin-sensitive neural pathways play in the pathogenesis of human disorders (Maggi and Meli, 1988; Maggi, 1991) will ultimately lead to a multipurpose use of improved vanilloids (synthetic resiniferatoxin analogs with advantageous spectra of action) in human therapy. Therefore, the emphasis of this article is on resiniferatoxin and on differences between RTX and capsaicin actions. Discussion of therapeutic indications of vanilloids in human disease states is focused on therapeutic potential, as suggested by pathophysiology, rather than on current use of capsaicin creams. Those who are interested in

Table I
Resiniferatoxin Potencies Relative to Capsaicin

	Source	Relative potency
High		
Twitch inhibition in rat vas deferens	Maggi *et al.*, 1990	10,000
Blocking acetic acid-induced writhings	Campbell *et al.*, 1989	6,000
Outflow of excitatory amino acids from rat spinal cord	Kangrga and Randic, 1991	5,000
Intermediate		
CGRP-LI[a] release from rat spinal cord	Maggi *et al.*, 1990	100
$^{45}Ca^{2+}$ uptake in cultured sensory neurons	Winter *et al.*, 1990	100
Depolarization of neonatal spine-tail preparation	Dray *et al.*, 1990	100
Low		
Depressor reflex in rabbit ear	Maggi *et al.*, 1990	3
Protective eye wipings in the rat	Szallasi and Blumberg, 1989	2
Contractions of rat urinary bladder	Maggi *et al.*, 1990	1

[a] CGRP-LI, calcitonin gene-related, peptide-like immunoreactivity.

the critical evaluation of current clinical uses of capsaicin are referred elsewhere (Carter, 1991; Rumsfield and West, 1991).

II. Vanilloid-Sensitive Neural Pathways and Their Role in the Pathogenesis of Disease States

The traditional separation of the peripheral nervous system into a somatic division composed of distinct afferent and efferent pathways and an autonomic division containing only efferents (Langley, 1921; Gabella, 1976) is inconsistent with recent findings that (1) peripheral nerves can mediate both afferent and efferent functions (Szolcsanyi, 1984), and (2) "autonomic" neurons can transmit nociceptive information into the central nervous system (Foreman *et al.*, 1986; Lembeck, 1988). There is an emerging concept of a unified network of both "somatic" and "autonomic" peripheral neurons that conducts physiological stimuli in a central direction and triggers reflex responses to maintain homeostasis (Prechtl and Powley, 1990). This network has been divided into two major neuronal populations (Prechtl and Powley, 1990):

1. The A neuron class appears early during ontogeny, is positive for the histochemical marker RT 97, gives rise to heavy myelinated (A-alpha and A-beta) fibers containing encapsulated receptors, and transmits predominantly proprioceptive and mechanoreceptive modalities.
2. The B neuron class appears late in ontogeny, depends on nerve growth factor during development, is negative for RT 97, possesses unmyelinated (C) or thin myelinated (A-delta) axons with bare receptors, and transmits nociceptive and thermoreceptive modalities (Fig. 2).

It is evident that both the A and B neuron classes are very diverse and the subdivisions made according to neurochemical, morphological, and electrophysiological criteria are of limited practical use (Lembeck and Bucsics, 1990). There is, however, general agreement that a major functional subpopulation of peripheral neurons (predominantly B-type) shares the trait of being susceptible to the triple (stimulatory/blocking/neurotoxic) action of capsaicin, the pungent ingredient in *Capsicum* (Buck and Burks, 1986; Holzer, 1991). The majority of these neurons are peptidergic, use adenosine or ATP as transmitter, and display high fluoride-resistant acid phosphatase activity (Buck and Burks, 1986). Nonetheless, there is no consensus as to the exact definition of being capsaicin-sensitive; that is:

1. One-third of the B neurons that are capsaicin sensitive at birth became capsaicin resistant during postnatal development (Lawson and Harper, 1984).
2. Capsaicin-sensitive nodose ganglion (vagal) neurons do not share the features, such as high substance P content or RT 97 negativity, that distinguish capsaicin-sensitive neurons in dorsal root ganglia (Dodd *et al.*, 1983; Dockray and Sharkey, 1986) and these neurons are in fact of different (placodal) origin. [*Note:* The fact that vanilloid sensitivity of neural pathways is linked to similarity in physiological functions rather than to embryonic origin strongly argues for the existence of endogenous modulators (endogenous vanilloids) that recognize the vanilloid receptor.]
3. Under certain experimental conditions capsaicin appears to act on A neurons, on retinal ganglion cells, as well as on neurons of the central nervous system, that is, on cells generally regarded as "capsaicin resistant" (Ritter and Dinh, 1988, 1990). Therefore, a number of terms and acronyms have been employed, such as CSA versus CSB (capsaicin sensitive in adult versus capsaicin sensitive in

Fig. 2 Classification of vanilloid-sensitive primary neuronal fibers.

newborn); capsaicin-sensitive sensory neuron with dual sensory-efferent function; capsaicin-sensitive primary afferent neuron; capsaicin-sensitive chemoreceptive B afferent, and so on (summarized by Szolcsanyi, 1984, 1990).

We use in the following the term *vanilloid-sensitive neural pathway* to describe any of those pathways that are either activated or inactivated by capsaicin and its analogs presumably in a receptor-mediated fashion, regardless of age, species, and embryonic origin; as a practical matter we are dealing with the vanilloid-sensitive peripheral (both B-type dorsal root and nodose ganglion) neurons of mature mammals. As a general rule, under physiological conditions this system transmits nociceptive and thermoceptive (heat) information to the central nervous system (Fig. 3) and triggers homeostatic reflexes regulating autonomic functions such as microcirculation (Holzer, 1988, 1991; Maggi, 1991); under pathological conditions this system transmits pain sensation and provokes "nocifensive" reflexes and neurogenic inflammation (Lembeck, 1983; Maggi and Meli, 1988; Holzer, 1991). Both peripheral and central nerve endings characteristically contain the tachykinin substance P (SP), which often coexists with other

Fig. 3 Simplified scheme of vanilloid-sensitive neural pathways.

neuropeptides, such as calcitonin-gene related peptide (CGRP) (Buck and Burks, 1986). On release SP preferentially binds to neurokinin 1 (NK-1) receptors (Guard and Watson, 1991) and thereby activates dorsal horn neurons involved in pain perception (Parris, 1991); in the periphery SP exerts diverse actions (Fig. 4) of which hypotension, smooth muscle responses, and increased vascular permeability (edema formation) are prominent examples (Holzer, 1988). Peripheral SP effects are mediated in part by histamine release from mast cells (Lembeck, 1983). Neuropeptides colocalized in vanilloid-sensitive neurons represent impressive examples for biological cooperativity; CGRP, for example, not only potentiates release of SP in the dorsal horn but also inhibits the endopeptidase that cleaves SP (Hokfelt, 1991).

Capsaicin actions show striking species differences. As a general rule, nonmammalian species are unresponsive to capsaicin actions (Jancso, 1968) [accordingly, we were unable to detect specific [^{3}H]RTX binding sites in sensory ganglia of chicken (Szallasi and Blumberg, 1990a)], whereas mammalian species can be classified as (1) sensitive both to

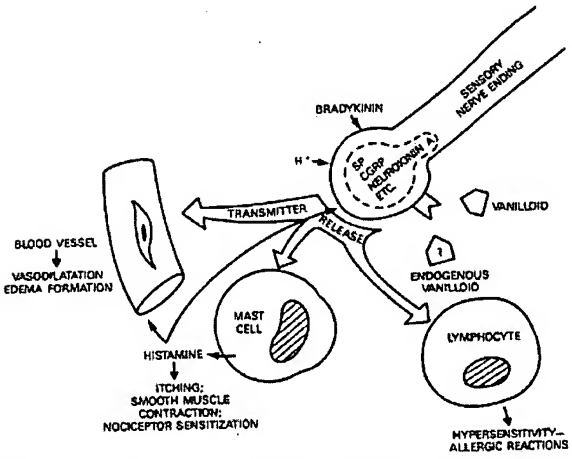


Fig. 4 Efferent function of vanilloid-sensitive nerve endings. SP, substance P; CGRP, calcitonin gene-related peptide.

irritation/desensitization and to respiratory depression by capsaicin (e.g., rat, guinea pig); (2) sensitive to irritation/desensitization but resistant to respiratory depression (e.g., hamster); and (3) sensitive to irritation only (e.g., rabbit) (Holzer, 1991). It has been speculated that the majority of the species differences are related to dissimilarities in tachykinin and CGRP levels in the nerve terminals (Maggi *et al.*, 1987; Amann *et al.*, 1988) or to a differential expression of neurokinin receptors on the target organs (Maggi, 1991). Nevertheless, there are still a number of puzzling species- and strain-related variations in the effects of capsaicin [e.g., intravenous capsaicin provokes the full pulmonary chemoreflex in dogs, cats, and Wistar rats, but not in Sprague–Dawley rats (Donnerer and Lembeck, 1982; Coleridge *et al.*, 1984; Adcock *et al.*, 1989)]; therefore, it is by no means easy to extrapolate capsaicin actions from animal experimentation to human beings. Experiments performed in healthy volunteers, however, suggest that capsaicin triggers the same reflex responses and provokes the same cutaneous reactions as in animals with distinct dose–response relations (Winning *et al.*, 1986; Bjerring, 1991; Fuller, 1990; Wallengren and Hakanson, 1992).

III. Mechanisms of Action of Vanilloids

A. Evidence for a Vanilloid Receptor

The selectivity of capsaicin action (cellular specificity, marked species differences) (Buck and Burks, 1986) and the fairly strict structural requirements for capsaicin-like activity strongly implied that capsaicin interacted at a specific site to produce its effects (Szolcsanyi and Jancso-Gabor, 1975, 1976). Direct evidence for a vanilloid receptor was furnished by the demonstration of specific [^{3}H]RTX binding by sensory ganglion and spinal cord preparations, which is fully competable by capsaicin (Szallasi and Blumberg, 1990a, 1991a). Corroborating indirect evidence comes from the reversible antagonism of capsaicin actions by capsazepine, a structurally related compound that lacks agonistic (stimulatory) activity (Bevan *et al.*, 1991; Dickenson and Dray, 1991; Urban and Dray, 1991).

1. Characterization of Specific Vanilloid Binding Sites

[^{3}H]RTX displays specific, saturable binding to membranes from both sensory ganglia and dorsal horn (Szallasi and Blumberg, 1990a, 1991a). Nonspecific binding, which is defined as that occurring in the presence of 100 to 1000 n*M* nonradioactive RTX, is not saturable and increases linearly with increasing concentration of the labeled compound. Scatchard analy-

sis of the data from filtration assays using rat dorsal root ganglion membranes fits the model of a single specific binding site possessing a K_d of 300 pM and a B_{max} of 150 fmol/mg protein (Szallasi and Blumberg, 1990a). Nonradioactive RTX displaced specific [^{3}H]RTX binding with a K_i of 110 pM, in fair agreement with the K_d value. Scatchard analysis of the data obtained in centrifugation assays, allowing direct measurement of free [^{3}H]RTX, gave binding parameters of K_d = 100 pM and B_{max} = 180 fmol/mg protein, respectively (Szallasi and Blumberg, 1992a). It should be noted that RTX is highly lipophilic and the resulting nonfavorable ratio of specific/nonspecific binding (at best, specific binding represents 50% of the total in rat dorsal root ganglion membranes) prevents the analysis of binding at very low or very high [^{3}H]RTX concentrations (Szallasi and Blumberg, 1990a). Consequently, based on the present binding assay, cooperativity or the existence of additional, quantitatively more minor binding sites cannot be ruled out.

At 37°C, specific [^{3}H]RTX binding is a time-dependent process attaining half-maximal binding within 2 min, reaching a plateau by 10 min, and maintaining this level for at least 60 min (Szallasi and Blumberg, 1991a). In keeping with the marked temperature dependence of *in vitro* capsaicin actions (Amann, 1990) reduced temperature (24°C) reduces the rate of specific [^{3}H]RTX binding [half-maximal binding appears by 10 min (unpublished observations)]. Whereas at 37°C association is too fast for quantification using current methodology, dissociation seems to be a complex process in which the dissociation rate changes as a function of incubation time in the presence of [^{3}H]RTX (Szallasi and Blumberg, 1992a). The underlying mechanism and the biological significance of the phenomenon remain to be determined.

The specific [^{3}H]RTX binding site is no doubt a protein: digestion by proteases or heating to 55°C completely abolishes binding activity (Szallasi and Blumberg, 1992a). Preliminary evidence suggests a role for –SH groups in ligand binding: SH-reactive heavy metal cations, such as Ni^{2+}, Hg^{2+}, and Cd^{2+}, block specific [^{3}H]RTX binding (unpublished observations). This finding might represent a mechanism by which these ions inhibit capsaicin actions in cultured neurons (Wood *et al.*, 1988).

Three major lines of evidence suggest that specific [^{3}H]RTX binding represents the vanilloid receptor:

1. *Pharmacological specificity:* Capsaicin and RTX structural analogs inhibit specific [^{3}H]RTX binding to both sensory ganglion and dorsal horn membranes in accord with their *in vivo* potencies; in contrast, unrelated compounds sharing some homology to the homovanillyl or to the diterpene moiety in vanilloids do not compete for specific [^{3}H]RTX binding sites (it is clear that the specific binding sites for [^{3}H]RTX are distinct from protein kinase C, the receptor for typical phorbol esters) (Szallasi and Blumberg, 1990a, 1991a; Szallasi *et al.*, 1991).

2. *Tissue specificity:* No specific [^{3}H]RTX binding was observed in neural tissues, such as cerebellum and ventral horn (Szallasi and Blumberg, 1990a), reported not to be affected by capsaicin (Jancso and Wolleman, 1977).

3. *Species specificity:* We found specific [^{3}H]RTX binding in sensory ganglia of several species, such as rat, mouse, hamster, guinea pig, cow, sheep, and pig (Table II) (Szallasi and Blumberg, 1990a, 1992a), in which specific vanilloid actions are well documented (Holzer, 1991), but not in chicken, in agreement with birds being unresponsive to the pungent activity of capsaicin (Jancso, 1968). Hamster and rabbit represent mammalian species comparatively insensitive to capsaicin (Glinsukon *et al.*, 1980). Our findings, however, indicate that the basic mechanism of vanilloid resistance differs dramatically between these two species: whereas the affinity and density of the vanilloid receptor in hamster trigeminal ganglia are comparable to those of the rat (Table II), no specific [^{3}H]RTX binding can be detected in the trigeminal ganglia of the rabbit (unpublished observations). These observations support the speculation that the relative capsaicin resistance in the hamster, but not in the rabbit, it related to low, almost undetectable substance P content in peripheral nerve terminals (Maggi *et al.*, 1987). Our current binding methodology allows us to detect specific [^{3}H]RTX binding in the

Table II

[^{3}H]Resiniferatoxin Binding in Sensory Ganglia of Different Species

	Source	K_d (nM)
Affinity determined		
Rat	Szallasi and Blumberg, 1990a	0.27
Guinea pig	Meini *et al.*, 1992	0.15
Pig	Szallasi and Blumberg, 1990a	2.2
Hamster	Szallasi and Blumberg, unpublished observation	0.2
Specific binding demonstrated		
Mouse	Szallasi and Blumberg, 1992a	—
Sheep	Szallasi and Blumberg, 1992a	—
Cow	Szallasi and Blumberg, 1992a	—
No specific binding found		
Chicken	Szallasi and Blumberg, 1990a	—
Rabbit	Szallasi and Blumberg, unpublished observation	—

perikarya and in the central terminals (dorsal horn), but not in the peripheral endings of vanilloid-sensitive neurons (Szallasi and Blumberg, 1992a). As different endpoints show differential dose requirements even in the same animal (Table I) (Szallasi and Blumberg, 1990b; Holzer, 1991), an unambiguous correlation between *in vivo* responses and *in vitro* vanilloid binding affinities cannot be made. There is, however, an overall agreement that in species such as guinea pig and rat, which are generally sensitive to the activation of vanilloid-like responses by RTX (Szallasi and Blumberg, 1990a; Franco-Cereceda *et al.*, 1991; Lou *et al.*, 1991), the affinity of the vanilloid receptor in sensory ganglia is higher than in species that are comparatively resistant, such as the pig (Franco-Cereceda, 1991; Szallasi and Blumberg, 1990a, 1991a; Meini *et al.*, 1992).

The apparent molecular size of an unpurified molecule can be determined by radiation inactivation analysis. The loss of biological activity (e.g., receptor–ligand binding) with increasing radiation doses allows the determination of the functional molecular target size (Harmon *et al.*, 1985). Analysis of loss of specific [^{3}H]RTX binding by high-energy irradiation yielded functional molecular masses of 270 kDa for pig dorsal root ganglion membranes and 280 kDa for pig dorsal horn preparation (Szallasi and Blumberg, 1991b). These sizes correspond to the functional molecular mass of other ligand-gated ion channels, such as the acetylcholine receptor/Na^+/K^+ ionophore complex (300 kDa) (Lo *et al.*, 1982) and the benzodiazepine/γ-aminobutyric acid receptor complex (220 kDa) (Chang and Barnard, 1982). A study using a capsaicin-like photoaffinity probe reported the specific labeling of two protein bands with molecular masses of 58 kDa and 42 kDa (Wood *et al.*, 1990). As these bands were also detected in dorsal root ganglia of chicken, a species insensitive to capsaicin actions (Juneso, 1968), and as capsaicin failed to inhibit labeling of these bands by the photoaffinity probe, we think that these bands most likely represent proteins specific to dorsal root ganglia but unrelated to the vanilloid receptor.

2. Capsazepine, a Functional Vanilloid Antagonist

Capsazepine has been shown to antagonize the effects of capsaicin without any detectable agonist activity in a variety of test preparations, including $^{45}Ca^{2+}$ uptake by cultured sensory neurons (Bevan *et al.*, 1991), C-fiber activation in saphenous nerve of the rat (Seno and Dray, 1991), depolarization of dorsal horn neurons of hemisected mouse spinal cord (Urban and Dray, 1991), and depolarization by noxious chemical and heat stimulation

of the ventral root in neonatal rat spinal cord preparation (Dickenson and Dray, 1991). Analysis of dose–response curves suggests a competitive mechanism: log dose–response curves are shifted to the right in parallel with increasing agonist (capsaicin) concentrations (Bevan *et al.*, 1991). It is, however, not known whether capsazepine displaces specifically bound [^{3}H]RTX; therefore the site at which capsazepine interferes with vanilloid actions remains uncertain. The *in vivo* use of ruthenium red, a nonspecific vanilloid antagonist with an obscure mechanism of action, is severely limited by its toxicity (Amann and Maggi, 1991). Whether capsazepine can be safely used in whole-animal studies remains to be evaluated.

B. Subcellular Mechanism of Action

Vanilloids are thought to open a voltage-insensitive (ligand-gated) cation channel (Fig. 5) that seems to be permeable for both monovalent and divalent cations with a limited selectivity for Ca^{2+} (Marsh *et al.*, 1987; Wood *et al.*, 1988; Bevan and Szolcsanyi, 1990). No secondary messenger

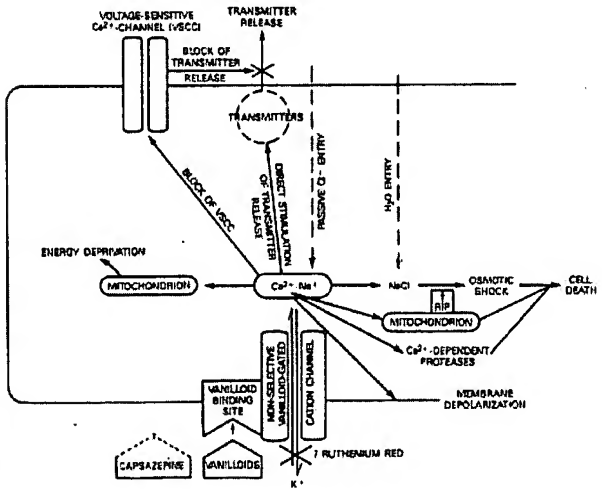


Fig. 5 Subcellular mechanism of action of vanilloids. RIP, rest in peace.

system appears to be involved in this action (Wood *et al.*, 1989). The apparent molecular mass of the vanilloid receptor (270–280 kDa) implicates a receptor complex that comprises at least one vanilloid-binding subunit and a channel; the complexity of this structure remains to be explored. It is unclear for example whether ruthenium red, an inorganic dye frequently used to block vanilloid actions both *in vivo* and *in vitro* (Amann and Maggi, 1991), acts on the channel or interacts at a distinct ruthenium red-sensitive subunit. Although this vanilloid-operated channel is frequently referred to as "novel," it might be an otherwise known channel gated by a modified (mutant) binding domain.

The net effect of Ca^{2+} and Na^{+} influx and K^{+} outflux is an inward current leading to membrane depolarization (action potential formation) and to direct stimulation of transmitter release by Ca^{2+} (Bevan and Szolcsanyi, 1990; Holzer, 1991). Persistent stimulation leads to high intracellular $[Ca^{2+}]$, which in turn activates Ca^{2+}-dependent enzymes and impairs mitochondrial functions (Fig. 5); these effects combined with the osmotic shock that follows intracellular NaCl accumulation can lead to irreversible damage and, ultimately, to cell death (Bevan *et al.*, 1987; Bevan and Szolcsanyi, 1990). This mechanism parallels glutamate-induced, NMDA receptor-mediated neurotoxicity (Choi, 1988; Komulainen and Bondy, 1988), linking vanilloids to the broader family of excitotoxins. The loss of response to further stimuli, generally referred to as *desensitization* (Jancso, 1968; Holzer, 1991), that follows excitation by vanilloids appears to be the net effect of diverse mechanisms, including

1. loss of vanilloid binding sites (Szallasi and Blumberg, 1992b);
2. inhibition of voltage-sensitive Ca^{2+} -channels by an unknown mechanism, which in turn blocks further transmitter release (Docherty *et al.*, 1991);
3. block of nerve conduction (Baranowski *et al.*, 1986);
4. block of both centripetal and centrifugal axonal transport, leading to nerve growth factor deprivation and contributing to the depletion of the releasable neuropeptide pool in nerve endings (Miller *et al.*, 1982; Taylor *et al.*, 1984, 1985); and
5. a general dysfunction of the affected neurons most likely the result of energy deprivation by impairment of mitochondrial functions (Joo *et al.*, 1969; Wood *et al.*, 1988).

The loss of responses is often divided into the subclasses of specific desensitization (i.e., desensitization that occurs on the receptor level) as opposed to nonspecific desensitization or, alternatively, (real) desensitization as opposed to defunctionalization (Holzer, 1991). In practice, vanilloid-induced desensitization is a mixture of the aforementioned mechanisms and possibly others; such classification is therefore of little relevance. We use, in the following, *vanilloid-induced desensitization* as a global term (Triggle, 1980) to describe any unresponsiveness to further stimuli if it follows vanilloid treatment and does not involve gross neurotoxicity.

IV. Therapeutic Potential of Vanilloids

A. Current and Potential Use of Vanilloids

The potential therapeutic uses of vanilloids are obviously as diverse as are the functions of vanilloid-sensitive neurons. Some uses, like analgesia, go back as early as the mid-1800s (e.g., mitigation of toothache by capsicum extracts); others, like neuromodulation of the immune system by vanilloids, are still in the embryonic stage. In theory, all three actions characteristic of vanilloids (stimulation, desensitization, neurotoxicity) may have therapeutic potential.

1. Stimulation

a. Counterirritancy Vanilloid-sensitive primary nociceptors enter the spinal cord via the dorsal root and synapse with second-order neurons located in layers I, II, and V of the dorsal horn (Fig. 3) (Rexed, 1952). In general, these second-order neurons can be classified either as nociceptive-specific (NS) or as wide dynamic range (WDR): NS neurons respond to noxious stimuli only, whereas WDR neurons, which also receive input from nonnociceptive afferents, respond to both innocuous and noxious stimuli (Willis, 1985). NS neurons in the marginal zone (lamina I) are major termination sites for A-delta mechanoheat-sensitive fibers; these neurons typically project to the contralateral midbrain and thalamus and are considered to be prime candidates for mediating pain sensation (Price and Dubner, 1977). In contrast, the substantia gelatinosa (lamina II) is the predominant termination site for unmyelinated C fibers and the majority of second-order lamina II neurons (both NS and WDR) project within the spinal cord. Lamina V neurons have a remarkably complex input, both direct from C and A-delta fibers and indirect from lamina I and II second-order neurons (Willis, 1985). It is essential to understand that for pain there is no linear sensory transmission system, but a complex dynamic interaction among ascending (including vanilloid-sensitive nociceptors) and descending (i.e., supraspinal) pathways that occurs predominantly in the dorsal horn, in part through excitatory and inhibitory intercalar neurons

(gate control theory of Melzack and Wall, 1965). Consequently, noxious stimulation of primary afferents of an organ, by closing the gate, can suppress the perception of the excitation of nociceptive afferents of another organ; this phenomenon, termed *nocigenic inhibition* (Ness and Gebhart, 1991) or *hyperstimulation analgesia* (Melzack, 1975), forms the scientific basis for acupuncture and counterirritation, used to relieve chronic pain since ancient times (Wand-Tetley, 1956).

Today the widest use of capsaicin in human therapy is as a counterirritant in topical nonprescription over-the-counter medications such as Omega Oil and Stimurub used to relieve muscle and joint pains. It is thought that the beneficial effect of these remedies is in part due to the increased cutaneous microcirculation (neurogenic flare) (Carter, 1991). Nonetheless, in these preparations vanilloids are not likely to have any advantageous effect as compared with other algesic substances.

b. Stimulation of Neuropeptide Release Vanilloids have contrasting effects on gastric ulcer formation depending on the route of administration: although systemic capsaicin was reported to aggravate gastric damage induced by intraperitoneal indomethacin or intragastric ethanol administration (Holzer and Sametz, 1986), topical (intragastric) capsaicin pretreatment was found to protect against gastric ulceration (Holzer *et al.*, 1987). Vanilloid-sensitive nerves are thought to play an essential role in the regulation of mucosal blood flow in the stomach (Lippe *et al.*, 1989). It is speculated that topical capsaicin releases calcitonin gene-related protein (Holzer, 1988) as well as a still unknown mediator enhancing prostaglandin formation (Uchida *et al.*, 1991), which in turn increases mucosal blood flow and thereby protects against gastric damage. This local neurogenic defense mechanism may also be activated by low pH (Geppetti *et al.*, 1990, 1991) but is impaired in animals desensitized to capsaicin (Holzer and Sametz, 1986). The pharmacological relevance of these observations is unclear. It is estimated that in hot climates the daily dietary intake of capsaicin may range from 0.5 to 1.0 mg/kg body weight (Monsereenusorn, 1983). Epidemiological study of this population may help to determine whether regular capsaicin consumption contributes to or protects against gastric ulcer formation in humans.

Limited experimental evidence suggests that locally applied capsaicin can accelerate wound healing in the pig (Watcher and Wheeland, 1989). The proposed mechanism is tachykinin release from the vanilloid-sensitive nerve endings which, in turn, can stimulate proliferation of both epithelial and mesenchymal cells (Sporn and Roberts, 1988). Although tachykinin depletion by systemic capsaicin has the reverse effect (Kjartansson *et al.*, 1987), vanilloid creams can still be safely used to optimize the healing process as they are unlikely to deplete neuropeptides (McMahon *et al.*, 1991). It has also been reported that the hyperinnervation of skin that follows a partial neuralectomy results in precocious hair development (Jones and Munger, 1987). Thus, a vanilloid lotion might be beneficial in the treatment of certain types of hair loss.

2. Desensitization

Vanilloids constitute a family of potential nonnarcotic, nonsteroidal analgesic/anti-inflammatory agents. Desensitization of vanilloid-sensitive neural pathways by topical capsaicin is already in use in human therapy; systemic desensitization by vanilloids represents a promising future research direction.

a. Analgesic/Antinociceptive Actions Neuropathic pain is defined as pain caused by the disruption and/or abnormal activity of primary afferent neurons in the absence of nociceptor stimulation (Bowsher, 1991). As has already been discussed in Section IV,A,1,a, if the afferent inhibition is decreased (as in entrapment neuropathies or after herpes zoster infection), nociceptive inputs can produce an exaggerated response which is often termed *deafferentation pain* (Bonica, 1990). Little is known about the abnormal activity of primary afferents: it is speculated that neuronal damage or the breakdown of isolation between adjacent axons can induce ectopic impulse generation in nociceptive neurons (Devor and Rappaport, 1990). Ectopic impulse generation by neuronal damage is supposed to play a central role in pain accompanying toxic and metabolic neuropathies such as diabetic neuropathy (Fields, 1987); the breakdown of isolation between axons can lead to "cross-talk" and is thought to form a major mechanism of pain production in demyelinization disorders (e.g., Guillain–Barré syndrome) as well as in reflex sympathetic dystrophy (Devor and Rappaport, 1990). Neurogenic pain syndromes are very common. They are estimated to have a prevalence of about 1% (Bowsher, 1991). Neuropathic pain is often persistent, is inappropriate to the existing illness (chronic pain syndrome) (Black, 1975), and cannot be managed without opioids (Portenoy, 1990). Therefore, vanilloids, which have been shown in animal experiments to selectively defunctionalize primary afferent pathways, have a most promising therapeutic potential in the management of neuropathic pain. Vanilloids, however, should be targeted to a site proximal to the site of the ectopic impulse generation, which requires either systemic administration or perineural infiltration, and capsaicin, as discussed in Section IV,B, cannot be safely administered to humans for this purpose.

Capsaicin creams (Axsain, Zostrix) have gained limited use in the treatment of pain in patients suffering postherpetic neuralgia (Watson, 1990), postmastectomy pain syndrome (Watson *et al.*, 1989), painful diabetic

neuropathy (Ross and Varipapa, 1989; Tandan *et al.*, 1992a,b; Capsaicin study Group, 1992), Guillain–Barré syndrome (Morgenlander *et al.*, 1990), reflex sympathetic dystrophy (Cheshire and Snyder, 1990), and vulvar vestibulitis (Friedrich, 1988). As follows from the pathomechanisms, the mechanism of action of topical capsaicin creams in the relief of neuropathic pain is speculative, and in some cases it is most likely restricted to a counterirritant action. In accord, a limited correlation has been found between the burning sensation that follows capsaicin treatment and the relief of neuropathic pain (Watson *et al.*, 1988). In contrast, capsaicin has been found to improve neuropathic pain even if the burning sensation was controlled by a local anesthetic ointment (Bernstein *et al.*, 1989). Hence, the value of capsaicin creams to relieve neuropathic pain is unclear (Dubner, 1991). Approximately 30% of the patients participating in the clinical trials decided to quit treatment because of the burning pain that followed treatment (Rumsfield and West, 1991), and the difference between capsaicin and placebo effects is hardly impressive: subjective improvement in approximately 60% of the capsaicin-treated patients versus a 30% improvement in placebo controls (Bernstein *et al.*, 1989).

Unlike neuropathic pain, itching (pruritus) is closely related to nociceptor activation in the periphery (Torebjork, 1974); its main mediator is presumably histamine (Bernstein, 1988). As was predicted (Toth-Kasa *et al.*, 1986), topical capsaicin very effectively relieves itching in a variety of disease states, including hemodialysis-related pruritus (Breneman *et al.*, 1992) and notalgia paresthetica (Wallengren, 1991).

b. Anti-inflammatory/Antiallergic Activity It is well documented that a variety of pain-producing, proinflammatory substances are stored in the peripheral terminals of primary afferent neurons (Lembeck, 1983; Buck and Burks, 1986; Holzer, 1988). The release of these substances from vanilloid-sensitive nerves is thought to play an important role in various disease states such as inflammatory diseases (including rheumatoid arthritis), in airway hyperreactivity/bronchial asthma, and in hyperreactive/allergic skin reactions (Maggi and Meli, 1988). Therefore, the block of release of these substances by vanilloid desensitization is expected to have therapeutic potential (Maggi and Meli, 1988). In fact, in animal models of human arthritis, systemic capsaicin was clearly beneficial (Colpaert *et al.*, 1983; Levine *et al.*, 1985). As it has been shown that inflammatory synovial fluid obtained from patients suffering from rheumatoid arthritis, similar to synovial fluid from rats with experimental arthritis, contains increased tachykinin-like immunoreactivity (Devillier *et al.*, 1986), a therapeutic potential for systemic vanilloid administration in humans is anticipated. Interestingly, substance P and neurokinin A, in sharp contrast to their lack of bronchoconstrictor activity in healthy volunteers, provoke severe bronchoconstriction in asthmatics (Joos *et al.*, 1986). The emerging concept of disease-related hyperreactivity to tachykinins (Barnes, 1986; Barnes *et al.*, 1991) suggests a further therapeutic application for the block of tachykinin release by vanilloid-induced desensitization.

In the mouse capsaicin has been found to block delayed-type hypersensitivity reactions (Girolomoni and Tigelaar, 1990); in the guinea pig and in the rat capsaicin pretreatment has been shown to mitigate ovalbumin sensitization (Nilsson *et al.*, 1991); and lymphocytes obtained from capsaicin-pretreated rats have been reported to secrete less immunoglobulins G and A than do lymphocytes of control animals following ovalbumin sensitization (Nilsson *et al.*, 1991). Neuromanipulation of the immune system by vanilloids thus represents a promising future research direction. It is also noteworthy that in normal rat intestine, mast cells are in a very close "beads-on-a-string" contact with primary afferent fibers (Bienenstock *et al.*, 1987); it remains to be determined whether in disease states characterized by increased numbers of intestinal mast cells, such as Crohn's disease and ulcerative colitis, desensitization to vanilloids can moderate symptoms.

Psoriasis is a skin disorder of unknown origin characterized by hyperplasia and altered differentiation of the epidermis, dilated capillaries in the dermis, and cutaneous inflammation (Farber *et al.*, 1986). Psoriatic lesions were shown to have a dense innervation of SP-positive nerve fibers (Naukkarinen *et al.*, 1989); increased SP synthesis and release are thought to play a central role in the inflammatory reaction (Farber *et al.*, 1986). Topical capsaicin application was found to somewhat improve psoriatic skin lesions (Bernstein *et al.*, 1986). As accidental surgical skin denervation has been reported to result in a dramatic improvement (Dewing, 1971), it is likely that the inefficacy of capsaicin creams can be attributed to inefficient drug delivery.

Apocrine chromhidrosis is a disorder of unknown etiology characterized by secretion of colored, usually blue or green, sweat in the axilla (Hurley, 1985). This disorder is rare but has no treatment (Hurley, 1985). Therefore the report of a successful suppression of chromhidrosis by topical capsaicin is of particular interest (Marks, 1989).

c. Improvement of Detrusor Instability The micturition reflex is regulated in part by tachykinin release from vanilloid-sensitive nerve endings (Maggi and Meli, 1988). Desensitization by capsaicin was shown to increase the volume threshold for initiating reflex micturia both in animal experiments (Maggi *et al.*, 1989b) and in patients suffering from hypersensitive bladder disorders (Maggi *et al.*, 1989a). Although the clinical trial was done on a very small group of patients, it is very promising that intravesical capsaicin infusion effectively relieved symptoms in patients

with severe hypersensitive bladder disorder refractory to any established treatment (Maggi *et al.*, 1989a).

d. Blocking Recurrent Genital Herpes Simplex Virus Infection Intraneural transport is the principal mechanism by which the herpes simplex virus (HSV) spreads within the host (Klein, 1982). Viral latency is established in dorsal root ganglia; recurrent infection occurs by the anterograde axonal transport of the reactivated virus (Klein, 1982). In the guinea pig, capsaicin pretreatment has been shown to ameliorate recurrent cutaneous herpetic disease (Stanberry, 1990).

3. Neurotoxicity

Rodents adequately tolerate the permanent loss of vanilloid-sensitive axons that follows perineural infiltration by high doses of capsaicin (Lynn *et al.*, 1987). Perineural infiltration with toxic vanilloid doses may be a promising approach for the mitigation of chronic, otherwise untreatable pain (Szolcsanyi, 1991).

B. Limitations on the Therapeutic Use of Capsaicin

Although the therapeutic potential of systemic (usually subcutaneous) capsaicin administration in animal experiments is well documented (Maggi and Meli, 1988), this approach has a number of limitations:

1. The therapeutic range of capsaicin given subcutaneously is very narrow: (a) In the rat capsaicin doses giving a half-effective desensitization against neurogenic (xylene-induced) inflammation result in respiratory depression, presumably via the activation of the pulmonary chemoreflex (Fig. 6) (Szolcsanyi *et al.*, 1991b); consequently, capsaicin desensitization requires either multiple treatments with increasing doses or respiratory assistance during treatment (Monsereenusurn *et al.*, 1982). (b) Therapeutically effective and neurotoxic doses overlap (Jancso *et al.*, 1985); moreover, high doses may have nonspecific toxic effects in the liver (Miller *et al.*, 1983), in smooth muscle cells (Monsereenusurn and Kongsamut, 1985), in the central nervous system (Ritter and Dinh, 1988), and in the retina (Ritter and Dinh, 1990), and may affect platelet function (Wang *et al.*, 1984).

2. Although the mechanism remains to be identified, capsaicin-treated animals are more sensitive to anesthesia which is used to mitigate the initial discomfort that follows capsaicin injection (Holzer, 1986).

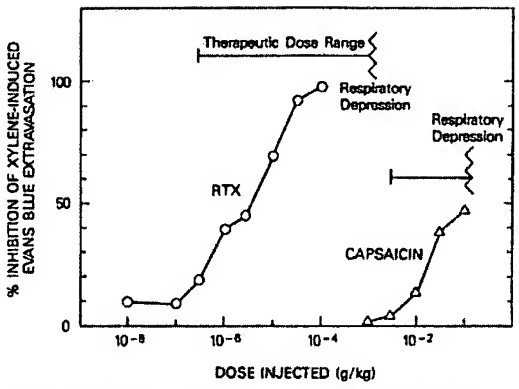


Fig. 6 Anti-inflammatory activity of resiniferatoxin (RTX) and capsaicin.

C. Improved Vanilloids

1. Resiniferatoxin

There is dramatic experimental evidence that these shortcomings of capsaicin can be reduced by using RTX, an ultrapotent vanilloid with a unique spectrum of actions (Szallasi and Blumberg, 1989; Szallasi *et al.*, 1989a; Szolcsanyi *et al.*, 1990, 1991b). Advantages of resiniferatoxin include (1) ultrapotency [sufficient long-lasting desensitization can be achieved by means of a single injection (Szallasi *et al.*, 1989a)] and (2) broad therapeutic range.

A most exciting feature of RTX is that it desensitizes several pathways with minimal or no excitation. A striking example is the lack of the activation of the triad of pulmonary chemoreflex (apnea, hypotension, bradycardia) by RTX (2 ng to 5 μg/kg IV) followed by desensitization of this reflex pathway to subsequent capsaicin and phenyldiguanide administration (Szolcsanyi *et al.*, 1990). As the activation of the pulmonary chemoreflex is supposed to play a central role in the respiratory depression that represents a major limitation for capsaicin administration (see preceding text), it is not surprising that rats can be fully desensitized to chemogenic pain and to neurogenic inflammation by single RTX doses that do not cause respira-

tory depression (Fig. 6) (Szolcsanyi *et al.*, 1991b). Other responses also show a very favorable excitation/desensitization ratio on RTX administration: RTX was found to have poor (capsaicin-like) potency in the chemogenic pain assay (protective eye wipings on ocular administration) (Szallasi and Blumberg, 1989) as well as in inducing contractions of isolated urinary bladders of the rat (Maggi *et al.*, 1990), although both responses were very effectively (at least 1000-fold more potently than capsaicin) desensitized by RTX.

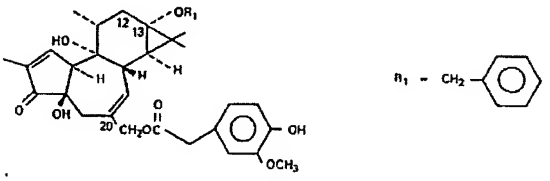
Although several studies have found neuron loss in rats treated with high doses of capsaicin (Jancso *et al.*, 1985; Chung *et al.*, 1985, 1990), in adult animals so far there is no evidence for RTX-induced neuronal loss (Szallasi *et al.*, 1989a; Szallasi and Blumberg, 1992b). Correspondingly, mature rats remain in apparent good condition after treatment with maximally effective RTX doses (Szallasi and Blumberg, 1992), unlike capsaicin-treated animals which, among other symptoms, frequently lose appetite, lose hair, acquire respiratory infections, and develop keratitis (Holzer, 1991).

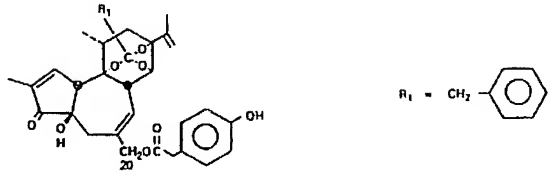
A possible explanation for these differences between the actions of capsaicin and RTX resides in the electrophysiological finding that whereas the capsaicin-induced inward ion current is rapid in onset and short in duration, the RTX-induced response, in contrast, develops slowly but is persistent (Winter *et al.*, 1990). RTX can thereby maintain a sustained Ca^{2+} current, which, in turn, can desensitize the neurons by an ill-defined mechanism without provoking action potentials or releasing mass amounts of transmitters (Szolcsanyi *et al.*, 1991a). It is obscure, however, why RTX and capsaicin, sharing the same binding site and ion conductance, act so differently at the single-cell level. On the whole-animal level, pharmacokinetic differences (a slower tissue penetration rate for RTX) (Maggi *et al.*, 1990) and receptor heterogeneity may also contribute.

2. Resiniferatoxin Structural Analogs

A promising future research direction is the evaluation of structure/activity requirements for RTX-like actions that might lead ultimately to the synthesis of rationally designed synthetic vanilloids with optimized spectra of actions. Limited analysis has already revealed the following:

1. Both the homovanillyl substituent and the diterpene moiety play an important role in the ultrapotency of RTX. If the homovanillyl substituent was missing (resiniferonol 9, 13, 14-orthophenylacetate, the 20-deacylated parent structure of RTX; see structure in Fig. 7) the affinity for the vanilloid receptor was lost (Szallasi and Blumberg, 1990a); likewise, this compound was found to be totally inactive for the





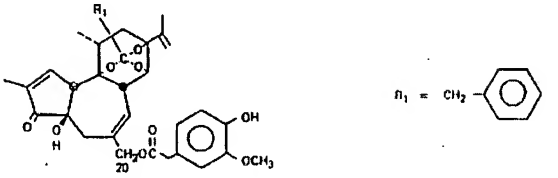


Fig. 7 Structures of resiniferonol 9,13,14-orthophenylacetate, resiniferatoxin, tinyatoxin, and 12-deoxyphorbol 13-phenylacetate 20-homovanillate.

biological responses characteristic of RTX (Szallasi *et al.*, 1989b); replacing the homovanillyl group with a 4-hydroxyphenyl group, such as found in tinyatoxin (Fig. 7), resulted in a moderate (three- to sixfold) decrease both in binding affinity and in biological potencies (Szallasi *et al.*, 1991). Modification of the diterpene moiety, for example, removal of the orthoester group such as found in 12-deoxyphorbol 13-phenylacetate 20-homovanillate (HV-dPP) (Fig. 7), dramatically reduced the binding affinity (Szallasi and Blumberg, 1991a), in keeping with HV-dPP being two to four orders of magnitude less active in the *in vivo* assays of inflammation and thermoregulation (Szallasi *et al.*, 1989b).

2. RTX structural analogs dramatically differ in their spectra of actions. There are two noteworthy examples: Tinyatoxin is practically inactive in the chemogenic pain (eye wiping) assay, although for other endpoints its potency is comparable to that of RTX (Szallasi *et al.*, 1991). HV-dPP, at doses that effectively block chemogenic pain and neurogenic inflammatory responses, fails to induce hypothermia (Szallasi *et al.*, 1989b). These RTX structural analogs imply that such undesirable RTX effects like pungency and hypothermia can be dissected by chemical modification.

V. Summary and Future Research Directions

Over the past three decades capsaicin has represented a useful tool to dissect a both morphologically and functionally diverse neural network that shares the trait of being susceptible to the stimulatory/blocking/neurotoxic effects of vanilloids; to identify the role of this network in maintaining the homeostasis of the organism and in contributing to disorders; and to predict the existence of a vanilloid receptor. Capsaicin, however, has failed to identify this receptor or to clarify its mechanistic pharmacology and has not fulfilled the expectations as a drug candidate.

The discovery of ultrapotent vanilloids, such as RTX, appears to give us a means to circumvent the shortcomings of capsaicin:

1. [^{3}H]RTX binding has already proved the existence of the predicted vanilloid receptor and provides a promising opportunity for the analysis of the pharmacology of the receptor, for the isolation/identification of the receptor, and for the detection of putative endogenous analogs.
2. The RTX class of vanilloids may further allow dissection of possible subclasses of vanilloid responses and may distinguish between specific and nonspecific effects.

3. The broad therapeutic range of RTX and its unique spectrum of actions make RTX a better drug candidate than capsaicin.
4. Structure/activity analysis of RTX analogs may lead to the synthesis of rationally designed vanilloids with optimized spectra of actions.

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[^{3}H]resiniferatoxin binding by the vanilloid receptor: species-related differences, effects of temperature and sulfhydryl reagents

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Summary. Specific binding of [^{3}H]resiniferatoxin (RTX) is thought to represent the vanilloid (capsaicin) receptor. In the present study, we have used this binding assay to elucidate the contribution of differential receptor expression to the capsaicin-resistance of hamsters and rabbits; binding parameters were compared to those of species (rats, mice) regarded as capsaicin-sensitive. Whereas the 5-fold lower affinity for [^{3}H]RTX binding in the hamster (100 pM) as compared to the rat (20 pM) is unlikely to account for the 100-fold difference in the in vivo responses of RTX-induced inflammation and hypothermia, the lack of detectable specific [^{3}H]RTX binding sites in the rabbit might represent the predominant mechanism of capsaicin-resistance in this species. Regulation of the vanilloid receptor was further characterized in the rat. In accord with the temperature dependence of both in vivo and in vitro capsaicin actions, we found a marked temperature dependence for association rates. Dissociation turned out to have complex kinetics dependent on time and receptor occupancy. Low pH (5.5 – 7.0) did not affect receptor binding. Preincubation with heavy metal cations and other sulfhydryl-reactive agents inhibited specific [^{3}H]RTX binding indicating that the vanilloid receptor is a thiol-protein, and that free sulfhydryl groups play an essential role in agonist binding activity. Preliminary characterization suggested noncompetitive inhibition.

Key words: [^{3}H]resiniferatoxin binding – Vanilloid receptor – Positive cooperativity – Species-related differences

Introduction

Primary afferent neurons, located in sensory (dorsal root and trigeminal) ganglia, receive information from the external and internal environment and transmit it to the central nervous system. A subclass of these neurons is activated by noxious chemical, thermal and mechanical stimuli (Szolcsanyi 1984; Baumann et al. 1991); is involved in the local regulation of vascular and immune reactions (Holzer 1988); and contributes to autonomic functions, such as micturition (Maggi and Meli 1986). Both afferent and efferent functions are mediated via the release of neurotransmitters, the best known example of which is substance P (Holzer 1988; Maggi 1991). These neurons are diverse in morphology and transmitter content (Lembeck and Buesies 1990) but have a common feature: after initial excitation they are inhibited by capsaicin, the irritant agent in hot peppers (Jancso 1960; Buck and Burks 1986). Neuroinhibition by capsaicin is generally referred to as desensitization, the terminology originally used by Jancso (1960). Recently, we have shown that resiniferatoxin (RTX), an irritant compound isolated from the latex of Euphorbia resinifera (Hergenhahn et al. 1975), acts as an ultrapotent capsaicin analog with a unique spectrum of actions (e.g. differentially effective for desensitization relative to pungency) (Szallasi and Blumberg 1989, 1990b). Since homovanillic acid constitutes the key motif both in capsaicin and RTX structural analogs, these bioactive compounds now appear to be best termed vanilloids.

Vanilloids are thought to interact at a specific receptor which, in turn, opens a nonselective cation channel (Bevan et al. 1987). The resulting cation influx (predominantly Na^+ and Ca^{2+}) leads to impulse generation and neurotransmitter release. Desensitization is most likely due to a combination of receptor loss (Szallasi and Blumberg 1992b), block of nerve conduction (Baranowski et al. 1986), inhibition of voltage-sensitive cation channels (Docherty et al. 1991), block of both anterograd and retrograd axonal transport (Miller et al. 1982), and a general dysfunction of the affected neurons which accompanies the mitochondrial damage by Ca^{2+} (Joo et al. 1969).

Vanilloids comprise a class of potential nonnarcotic, nonsteroidal analgesic-antiinflammatory agents (Maggi

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and Meli 1988). Topical capsaicin creams are already in use, but as yet their efficacy is inconclusive (Carter 1991; Rumsfield and West 1991). Animal experimentation suggests that systemic capsaicin might be a powerful drug to relieve a number of chronic pain conditions and inflammatory disorders (Maggi and Meli 1988). However, it is complicated extrapolating to humans since in animals capsaicin shows striking species-related differences in action. For example, the guinea-pig, a species shown to be very susceptible to capsaicin actions (Glinsukon et al. 1980; Buck et al. 1983), is frequently used as a model of bronchial asthma although the sensory innervation of the human lung is rather "rabbit-like" (Laitinen et al. 1983), and rabbits, in sharp contrast to guinea-pigs, are very resistant to capsaicin (Glinsukon et al. 1980). Species-related differences in vanilloid-sensitivity may reflect (1) differences in the innervation of organs, (2) differences in neurotransmitter levels and/or pattern in vanilloid-sensitive nerve endings, (3) a differential pattern of receptors for these neurotransmitters, (4) differential expression of the vanilloid receptor and/or receptor heterogeneity, and (5) differences in second messenger mechanisms.

Whilst the contributions of differences in neuropeptide levels (Maggi et al. 1987; Geppetti et al. 1988) and in neuropeptide-receptor expression (Guard and Watson 1991) to vanilloid-sensitivity are beginning to be understood, very little is known about the role that the vanilloid receptor plays in determining the vanilloid-sensitivity of a species. In the present study, we have therefore characterized specific [^{3}H]RTX binding sites, thought to represent the vanilloid receptor (Szallasi and Blumberg 1990a, 1992a), in mammalian species regarded as capsaicin-sensitive (rat; mouse) and capsaicin-resistant (hamster; rabbit). We have also assayed for specific RTX binding in alligators, since reptiles, although they are not supposed to express vanilloid receptors (Holzer 1991), were reported to respond to capsaicin administration (Kanui et al. 1990). In addition, we have examined the effect of protons (Bevan and Yeats 1991), thiol-reagents (Evangelista et al. 1992), and low temperature (Szolcsanyi 1977; Amann 1990), factors shown to regulate vanilloid-sensitive neurons, on the vanilloid receptor.

Methods

Sprague-Dawley rats (females, 200–250 g), CD-1 mice (females, 18–20 g), syrian golden hamsters (females, 175–200 g), and New Zealand White rabbits (females, 2–3 kg) were used. Alligators (approximately 30 cm long) were purchased from Gator Jungle, Plant City, Fla., USA. Animal experimentation was performed in accord with the Guide for the Care and Use of Laboratory Animals (DHEW Publication No. (NIH) 80-23, DRR/HIH, Bethesda, Md., USA, 1980) and was approved by the institutional animal use committee.

For binding assays (Szallasi and Blumberg 1990a, 1992a), animals were sacrificed by decapitation under CO_2 anesthesia, and the cranial and cervical spinal sensory ganglia were removed and disrupted by the aid of a Polytron tissue homogenizer in ice-cold buffer A (pH 7.4) containing (in mM): KCl 5, NaCl 5.8, $MgCl_2$ 2, $CaCl_2$ 0.75, sucrose 137, and HEPES 10. Tissue homogenates were then washed twice with the same buffer and the particulate fraction stored at −70°C until assayed. Binding assays were carried out in a volume of 0.5 ml containing buffer A, 0.25 mg/ml bovine serum albumin (Cohn fraction V; Sigma, St. Louis, Mo., USA), labelled and nonradioactive RTX, and, unless specified otherwise, 30–40 µg of particulate fraction protein. Tubes were kept on ice while the additions were made. The assay mixture was incubated for 30 min at 37°C. After the binding reaction had been terminated by cooling the tubes on ice, 100 µg alpha$_1$-acid glycoprotein (AGP; Sigma, St. Louis, Mo., USA) in 50 µl of buffer A was added to each tube to reduce nonspecific binding. [AGP is a plasma protein that binds RTX at 0°C with an affinity of 330 nM (Szallasi et al. 1992). Nonspecifically bound RTX redistributes between AGP and the membrane lipids, thus the amount of nonspecifically bound RTX that remains in the membranes is diminished. Since at 0°C the release of receptor-bound RTX is unmeasurably slow, by using AGP we were able to reduce nonspecific binding by rat dorsal root ganglion membranes from 50% to 10–20% of the total binding without compromising specific binding (Szallasi et al. 1992).] Bound and free [^{3}H]RTX were then separated by pelleting the membranes in a Beckman 12 microfuge. The tip of the microfuge tube containing the pelleted membranes was cut off with a razor blade, and the bound radioactivity determined by scintillation counting. Points were determined at least in triplicate; each binding experiment was done at least twice. Nonspecific binding was defined as that occurring in the presence of 100 nM nonradioactive RTX; its contribution to total binding is given for each species in the Results. Binding was expressed as fmol/mg membrane protein. Protein was determined using the Bio-Rad protein assay.

The range of [^{3}H]RTX concentrations used for the analysis of binding parameters was 6–400 pM for the mouse and rat and 12–800 pM for the hamster. Typically, at the lowest [^{3}H]RTX concentration there were approximately 50 dpm/pellet, including a machine background of 20 dpm and 2–4 dpm of non-specific binding. Due to the low level of bound radioactivity, samples were equilibrated for at least 12 h before counting and were then counted for 10 min each. At 400 pM [^{3}H]RTX the total activity in the pellet was approximately 600 dpm, including 300 dpm of non-specific binding.

Binding data from saturation experiments using increasing concentrations of [^{3}H]RTX were analyzed by computer fit to the Hill equation (Endrenyi et al. 1975).

$$B = (B_{max} \cdot L_H^n)/(K_d^n + L_H^n)$$

where B represents specifically bound [^{3}H]RTX, B_{max} is the receptor density, L_H is the concentration of free [^{3}H]RTX, K_d is the concentration of [^{3}H]RTX at which half of the receptors are occupied, and n is the cooperativity index frequently referred to as the Hill coefficient (Endrenyi et al. 1975). If $n = 1$, receptors are independent of each other; $n < 1$ indicates negative cooperativity, and $n > 1$ implies positive cooperativity among binding sites. The Hill equation predicts that the typical linear Scatchard plot that characterizes no cooperativity becomes concave in case of negative cooperativity and convex in case of positive cooperativity (Endrenyi et al. 1985). The data were fitted to the Hill equation using the program Fit P (Biosoft, Milltwon, N. J., USA).

Association of [^{3}H]RTX (25 pM, a concentration close to the K_d value of 18 pM; Results) to the specific binding sites was followed between 2–60 min at 4°C, 21°C, and 37°C. Nonspecific binding in the presence of 100 nM nonradioactive RTX was determined for each time point.

Dissociation was determined as follows: after steady state binding had been achieved (10 min at 37°C; as determined in the association experiments) AGP (600 µg/tube) was added to prevent rebinding of released RTX, and the incubation continued at 37°C for an additional 5–120 min. Control binding was determined for each time point in replicate sets of tubes. Nonspecific binding was independent of the incubation time; it was usually determined three times during the experiment, and the average of the three determinations was used for the calculations.

For chemical modification, membrane suspensions in buffer A were incubated with the indicated concentrations of sulfhydryl-

reactive reagents for 30 min at room temperature (21°C). Then the binding reaction was initiated by adding 25 pM [^{3}H]RTX with or without nonradioactive RTX to determine nonspecific binding; the tubes were warmed to 37°C; and the assay mixture incubated for 10 min at 37°C.

pH-dependence of the binding was determined using a filtration assay performed as already described (Szallasi and Blumberg 1990a). Binding was determined in the range of pH 5.5–9.0 using the following buffers (Sigma, St. Louis, Mo., USA): 0.1 M MES; pH 5.5–6.5; 0.1 M BIS-TRIS, pH 6.0–7.0; 0.1 M HEPES, pH 7.0–8.0; 0.1 M TRIZMA, pH 8.0–9.0. Binding was determined using 60 pM [^{3}H]RTX.

Inflammatory activity of RTX in rats, mice, and hamsters was determined by measuring tissue swelling 30 min after the compound had been applied to the inner surface of the right ear in a volume of 20 µl acetone. The left ear of the same animals served as a solvent control. Animals were euthanized by CO_2; the ears were removed; and tissue plugs obtained from the tips of the ears were weighed. Dose response curves were performed with 1:3 dilutions of RTX. For the mouse and rat, 8–10 animals were tested at each RTX dose. For the hamster, 4–6 animals were tested at each dose.

To induce hypothermia, RTX at the indicated doses was injected s.c. in a volume of 50 µl EtOH at the back of the neck of animals under mild ether anesthesia. Rectal temperature 1 h prior to and after injection was determined by a small-animal temperature probe (Cole-Parmer, Chicago, Ill., USA) (Szallasi and Blumberg 1989). During the experiment, animals were kept in an environment at a regulated temperature of 20°C.

[^{3}H]RTX (37 Ci/mmol) was synthesized by the Chemical Synthesis and Analysis Laboratory; NCI-FCRDC (Frederick, Md., USA). Nonradioactive RTX was purchased from Chemicals for Cancer Research, Inc. (Chaska, Minn., USA) and from Chemsyn Science Laboratories (Lenexa, Kan., USA). NEM and DTNB were from Sigma, St. Louis, Mo., USA. PCMB was from ICN-Flow, Costa-Mesa, Calif., USA.

Results

Specific [^{3}H]RTX binding in rats, mice, hamsters, rabbits, and alligators

In saturation experiments using increasing concentrations of the labelled ligand, [^{3}H]RTX displayed specific, saturable binding to sensory ganglion membranes of the mouse and hamster; we had observed this behaviour previously for the rat (Szallasi and Blumberg 1990a) and we confirmed it here for the rat in the present series of experiments. Nonspecific binding was linear with the concentration of the labelled compound and, at the concentration that saturated specific binding sites, represented 20% of the total (not shown). Kinetics of specific binding was sigmoidal indicating positive cooperativity (not shown). The binding parameters were calculated by fitting the measured data to the Hill equation (see Methods). The affinity of the vanilloid receptor was similar in the rat and in the mouse (18 ± 2 pM and 22 ± 5 pM, respectively; 2 determinations; mean ± range), but was almost 5-fold lower in the hamster (97 ± 25 pM; 2 determinations; mean ± range). The cooperativity index was 1.6 ± 0.2 in the rat, 1.9 ± 0.3 in the mouse, and 1.4 ± 0.2 in the hamster. The values in the rat agree well with those we determined previously (K_d = 24 pM; n = 1.7; Szallasi and Blumberg submitted). The receptor density was 78 ± 15 fmol/mg protein in the rat, 31 ± 6 fmol/mg protein in the mouse, and 75 ± 10 fmol/mg protein in the hamster. Figure 1 shows the Scatchard plots of the data from the saturation experiments; due to the positive cooperativity all three Scatchard plots are convex; on the other hand, whilst the shapes of the Scatchard plots are similar in the rat and in the mouse as anticipated by the similar K_d and n values, in the hamster the Scatchard plot appears flatter due to a combination of lower affinity and less pronounced cooperativity. In contrast to the results with these three species, no specific binding could be detected in the rabbit and in the alligator (2 tissue preparations each, data not shown).

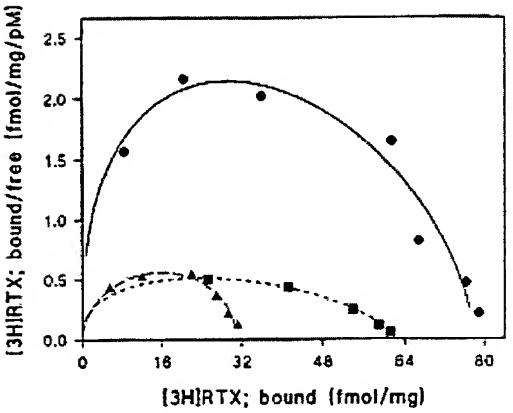

Fig. 1. Scatchard plot of specific [^{3}H]RTX binding by particulate preparations obtained from sensory ganglia of the rat (●), mouse (▲), and hamster (■). *Points* represent mean values from a single experiment; theoretical *curves* were fitted to the measured data by using B_{max} and K_d values, as well as Hill numbers determined by computer fit to the Hill equation (see Methods). A second experiment gave similar results

Inflammatory and hypothermic activity of RTX in rats, mice, and hamsters

In order to correlate the in vitro receptor binding affinities to the in vivo potencies, we determined the inflammatory and hypothermic potencies of RTX in the rat, in the mouse, and in the hamster. For the induction of ear edema, RTX displayed similar potencies in the rat and in the mouse (estimated EC_{50}'s were 1 µg/ear in the rat, and 3 µg/ear in the mouse) whilst in the hamster RTX, up to 80 µg/ear, did not cause a measurable edema response (not shown). Likewise, RTX induced hypothermia with similar ED_{50}'s in rats and mice (2×10^{-7} g/kg and 10^{-6} g/kg, respectively) but was 2 orders of magnitude less potent in the hamster ($ED_{50} = 10^{-4}$ g/kg) (Fig. 2). The results on hypothermia in the rat and mouse agree with previous measurements (DeVries and Blumberg 1989; Szallasi and Blumberg 1989). Vanilloids are thought to excite the hypothalamic heat center, which, in turn, activates the heat loss mechanisms of the species (Szolcsanyi et al. 1971). Under these conditions, heat loss is a function of the relative body surface. The relative body surface was similar in rats and hamsters employed in our experiments; in accord, the maximal response was

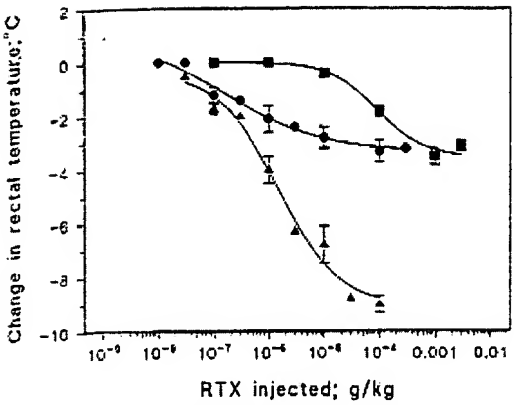

Fig. 2. Induction of hypothermia by RTX in the rat (●), in the mouse (▲), and in the hamster (■). The fall in rectal temperature was measured 1 h after s.c. injection of RTX at the indicated doses. Each point represents 5–10 animals; mean ± SEM

similar in these two species (−3°C; Fig. 2). Mice have a bigger relative body surface; consequently, RTX produced a bigger drop in rectal temperature (−9°C) in this species (Fig. 2), as observed previously (DeVries and Blumberg 1989).

On-rate and off-rate of [³]RTX: effects of temperature and receptor occupancy

Inhibition of capsaicin action in vivo by low temperature is well documented (Szolcsanyi 1977; Amann 1990). Although this phenomenon has not yet been well characterized, the observation that the response at low temperatures can be restored by increasing the concentration of capsaicin (Amann 1990) implies diminished binding rate or affinity as a possible mechanism. To explore this hypothesis, we have determined the temperature-dependence of the association of RTX with specific binding sites in sensory ganglia of the rat (Fig. 3).

In accord with our previous observations (Szallasi and Blumberg 1990a), at 37°C the on-rate of [^{3}H]RTX was fast; specific binding at 25 pM [^{3}H]RTX had attained 50% of its final value within minutes and then remained on an essentially constant level over the 60 min incubation. In contrast, at 24°C specific [^{3}H]RTX binding showed a gradual increase during the time course of the experiment and specific binding reached only 50% of the steady state binding characteristic of 37°C by the end of the 60 min incubation (Fig. 3). At 4°C the association of the labelled compound became undetectably slow (Fig. 3).

An unexpected result of ours is shown in Fig. 4. Using a fixed (25 pM) concentration of [^{3}H]RTX, the fraction of the labelled compound that could be released (dissociation) during a 10 min incubation period was a function of the incubation time allowed for association. Whereas 80% of [^{3}H]RTX bound during the first 2 min of association was released by adding AGP to the assay mixture and continuing the incubation for an additional

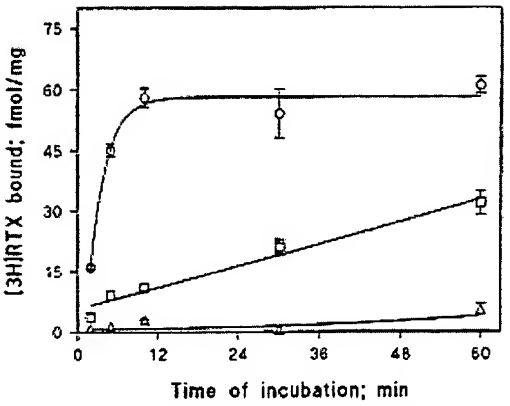

Fig. 3. Association of [^{3}H]RTX (25 pM) to specific binding sites in sensory ganglia of the rat at 37°C (○), 24°C (□), and 4°C (△). *Points* are from a single determination done in triplicate; mean ± SEM. The association rate at 37°C was measured 5 additional times with similar results; the on-rates at 24°C and at 4°C were confirmed in a second experiment. Curves were fitted using the exponential sigmoid equation

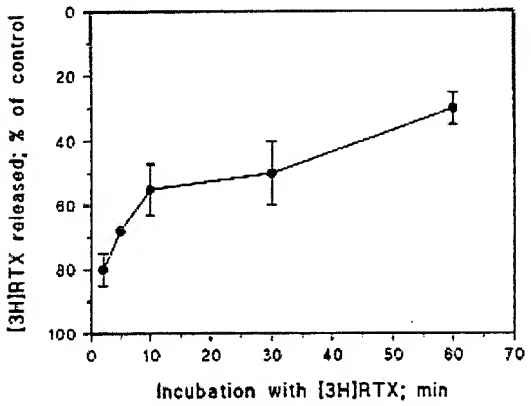

Fig. 4. [^{3}H]RTX released during the first 10 min of dissociation as a function of time allowed for association. The determination was done 4 times; data are from a representative experiment. 100% values were determined for each time point; release was induced by preventing the rebinding of [^{3}H]RTX by adding alpha$_1$-acid glycoprotein, a plasma protein that binds RTX, to the assay mixture

10 min, less than 30% of the labelled compound that bound during a 60 min association was dissociated from the receptor under similar experimental conditions (Fig. 4). These observations may imply a time-dependent and presumably ligand-induced conformational change of the receptor which leads to slower dissociation rate. In accord with this assumption, following a 10 min incubation with 6 pM [^{3}H]RTX, a concentration estimated to occupy only 20% of the receptors, the off-rate was fast: 80% of the bound compound was released within the first 5 min of the dissociation experiment (Fig. 5). On the other hand, if dissociation was initiated at an estimated 50% receptor occupancy (10 min incubation with 25 pM [^{3}H]RTX), the off-rate became slower: only 30% of the bound ligand was released during the first 5

(Szolcsanyi 1984; Buck and Burks 1986; Holzer 1991). In fact, the classification of primary afferent neurons is usually based on capsaicin-sensitivity (Jancso 1990). However, there seems to be no consensus as to the exact definition of being "capsaicin-sensitive": (1) in neonates more neurons are sensitive to neurotoxicity by capsaicin than in adults; (2) in adults high doses of capsaicin apparently affect neurons that do not fit any morphological or functional criteria shared by neurons sensitive to lower capsaicin doses, and high doses of capsaicin affect nonneural tissues; (3) the species and strain differences in capsaicin sensitivity are so striking that it is not possible to predict the spectra of capsaicin actions in a given species or to extrapolate from animals to humans (see Holzer 1991 for review). It is, however, generally accepted that "specific" capsaicin actions are mediated via a specific capsaicin (vanilloid) receptor which is expressed only on B-type (small) primary afferent neurons of mammals, whereas "nonspecific" effects are caused either by interaction with other receptors or by non-receptor-mediated mechanisms (cf. Holzer 1991). The experimental evaluation of this view has only recently become possible with the discovery of ultrapotent capsaicin analogs (cf. Szallasi and Blumberg, 1990b), the paradigm of which is resiniferatoxin (RTX), and the resulting availability of a biochemical means to identify specific vanilloid binding sites (Szallasi and Blumberg 1990a, 1992a). In the present study, we have utilized the [^{3}H]RTX binding assay to determine whether or not the capsaicin resistance of the hamster and the rabbit (Glinsukon et al. 1980; Maggi et al. 1987) is due to a differential expression of vanilloid receptors as compared to rats and mice, species sensitive to capsaicin actions (cf. Buck and Burks 1986).

The rat and the mouse, in accord with the similar overall capsaicin-sensitivity of these species, showed similar binding affinities for RTX. Likewise, the cooperativity indices for RTX binding were similar. In contrast, vanilloid receptors in the hamster bound RTX with a 5-fold lower affinity, and the cooperativity index was also lower. Nonetheless, these differences in properties of vanilloid receptors in rats and hamsters are unlikely to account for the dramatic (100-fold) difference in potencies of RTX to induce inflammation and hypothermia in these two species. We conclude that, although the lower affinity of the vanilloid receptor might contribute to the relative vanilloid-resistance of hamsters, other species-related differences like the very low tachykinin levels in vanilloid-sensitive nerve endings (Maggi et al. 1987), metabolism, or pharmacokinetics play the predominant role.

We were unable to detect specific [^{3}H]RTX binding sites in trigeminal ganglia of the rabbit. Low density and/or low affinity of vanilloid receptors in the rabbit may therefore be the predominant factor in the very marked resistance of this species to vanilloid actions (Glinsukon et al. 1980). Unlike hamsters, rabbits have tachykinin (substance P) levels in the bladder similar to that of rats (Maggi et al. 1987). It has been reported that sufficiently high doses of capsaicin contract isolated rabbit bronchi in vitro (Spina et al. 1991) and increase blood flow in rabbit skin in vivo (Buckley et al. 1990). The latter response is inhibited by Ruthenium Red (Buckley et al. 1990). The probable explanation is that rabbits possess vanilloid receptors but their expression remains below the detection limit of the present [^{3}H]RTX binding assay because of low affinity, possibly coupled with low receptor number.

Since a basic criterion of "specific capsaicin action" for decades has been that the response is restricted to mammals (cf. Jancso et al. 1967; Jancso 1968; Holzer 1991), the recent report of a supposed pain response (eyeball-rollings and eye-closing) in the crocodile (Kanui et al. 1990) was unexpected. Since we could not repeat this response in alligators, another reptile species, nor could could we detect specific [^{3}H]RTX binding in the cranial sensory ganglia of the same animals, the specificity of capsaicin-induced eye reactions in the crocodile requires replication before it is accepted.

A striking feature of capsaicin actions is their very marked dependence on temperature. Cooling the skin or isolated organs to room temperature (18–20°C) attenuates both excitation and desensitization by capsaicin (Szolcsanyi 1977; Amann 1990). In accord, we found that the association rate of [^{3}H]RTX is slowed dramatically at 24°C. Until the relationship is clarified between binding and stimulation on the one hand, and desensitization by vanilloids on the other hand, the suggestion of Dray and coworkers that there exists a distinct mechanism of stimulation by capsaicin that does not lead to desensitization (Dray et al. 1989) remains uncertain since it is based on experiments performed at 20°C.

Whereas at 37°C association is too fast for quantitation using our current methodology, dissociation seems to be a complex process in which the dissociation rate changes both as a function of fractional receptor occupancy and as a function of incubation time in the presence of [^{3}H]RTX under steady-state conditions. The detailed quantification, underlying mechanism(s), and biological significance of this phenomenon remain to be determined.

A major finding of the present study is that free sulfhydryl groups are essential for ligand-binding activity of the vanilloid receptor. The alternative explanation, that the reagents affect the [^{3}H]RTX directly, can be excluded since pre-incubation of the membranes is required to give inhibition. The vanilloid receptor thus resembles the brain dopamine D1 receptor (Braestrup and Andersen 1987), the brain glutamate receptor (Kiskin et al. 1986), and the brain muscarinic acetylcholine receptor (Aronstam et al. 1978). This finding provides a mechanism to account for the observations that the free sulfhydryl alkylating agent NEM can block capsaicin-induced CGRP-release in the stomach (Evangelista et al. 1992), and sulfhydryl-reactive heavy metal cations can inhibit capsaicin-induced responses in cultured DRG neurons (Wood et al. 1988).

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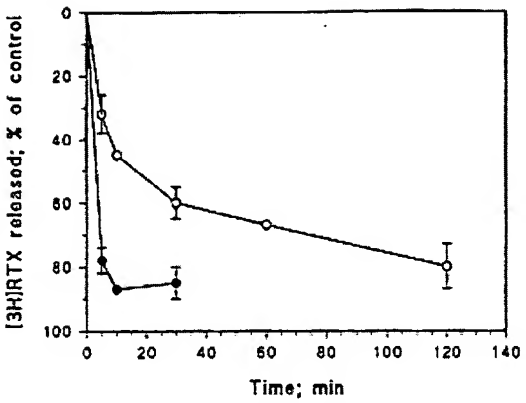

Fig. 5. Dissociation of [^{3}H]RTX as a function of fractional receptor occupancy. Preparations from rat sensory ganglia were incubated for 10 min at 37°C with 6 pM (●) or 25 pM (○) [^{3}H]RTX; these concentrations yield estimated receptor occupancies of 15% and 62%, respectively. Then, at the 0 min time point alpha$_1$-acid glycoprotein (AGP), a plasma protein that binds [^{3}H]RTX, was added to prevent rebinding of the dissociating labelled compound. % release was calculated from specific [^{3}H]RTX values determined in the presence or absence of AGP for each time point. The experiment was performed 3 times; data are from a representative experiment

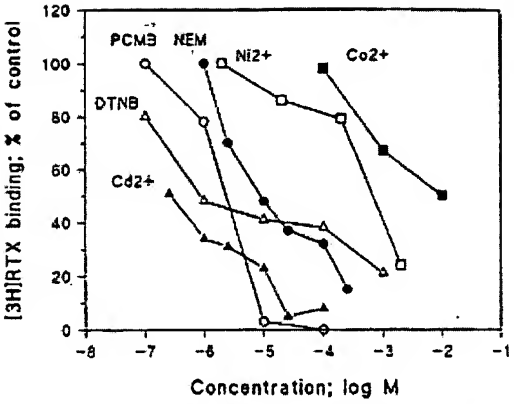

Fig. 6. Inhibition of specific [^{3}H]RTX binding by N-ethylmaleimide (NEM), p-chloromercuribenzoate (PCMB), 5,5′-dithio-bis-2-nitrobenzoate (DTNB), Cd^{2+}, Ni^{2+}, and Co^{2+}. Rat sensory ganglion membranes were preincubated with the indicated concentrations of the above agents for 30 min at 24°C, then the binding reaction was initiated by adding 25 pM [^{3}H]RTX, and specific binding was determined following a 10 min incubation at 37°C. *Points* represent the average of 2 determinations for NEM and Co^{2+}, and the average of 3 determinations for PCMB, DTNB, Cd^{2+}, and Ni^{2+}

min of the dissociation experiment and the 80% level of release was not achieved earlier than 120 min (Fig. 5).

Effects of chemical modification of the vanilloid receptor: pH, heavy metal cations and other thiol-reagents

Low pH, which can occur under pathophysiological conditions, has been shown to activate vanilloid-sensitive neurons (Bevan and Yeats 1991; Geppetti et al. 1991), and has been speculated to represent an endogenous analog for the vanilloids (Bevan and Yeats 1991). To avoid inactivation of AGP by protons, the effect of pH (5.5–9.0) on specific [^{3}H]RTX binding has been examined in our standard filtration assay without AGP (Szallasi and Blumberg 1990a). In the range of 5.5–7.5 an essentially constant level of specific binding was found. Binding was reduced by 50% at pH 8.0 and by 70% at pH 9.0.

Very recently, it has been found that N-ethylmaleimide (NEM), an agent that alkylates sulfhydryl groups, first stimulates and then blocks release of CGRP-LI from rat stomach (Evangelista et al. 1992). The cross-tachyphylaxis between NEM and capsaicin actions suggests that NEM recognizes the vanilloid receptor, or, in other words, that the vanilloid receptor is a thiol-protein. Inhibition of a receptor function by NEM increases with the time of exposure (Karlin and Bartels 1966). In a preliminary experiment, rat sensory ganglion membranes were therefore preincubated with 100 µM NEM (the concentration employed by Maggi's group in the rat stomach experiments) at 37°C for 5–60 min, and then specific [^{3}H]RTX binding was determined during an additional 10 min incubation. It turned out that specific [^{3}H]RTX binding diminished in the membranes preincubated with NEM: the inhibition reached its plateau value (20% of residual [^{3}H]RTX binding) by 20 min preincubation (not shown). Therefore, in the experiments detailed below a standard preincubation of 30 min were employed in the presence of the thiol-modifying agents. Inhibition by NEM was concentration-dependent with an approximate IC_{50} of 10 µM (Fig. 6). Other free thiol group reactive agents, such as p-chloromercuribenzoate (PCMB) and 5,5′-dithio-bis-2-nitrobenzoate (DTNB also known as Ellman's reagent), also inhibited specific [^{3}H]RTX binding (Fig. 6). Among the sulfhydryl-reactive heavy metal cations the rank or order of potency to inhibit the vanilloid receptor was $Cd^{2+} \gg Ni^{2+} > Co^{2+}$ (Fig. 6). Other divalent cations, like Ca^{2+} and Mg^{2+}, up to 10 mM failed to block RTX binding (not shown). The anion for all cations tested was Cl^-. The nature of the inhibitory effect of DTNB and Cd^{2+} was investigated by saturation analysis of [^{3}H]RTX binding in the absence or presence of these agents (10 µM DTNB; 1 µM Cd^{2+}). Although the low amount of specific binding measured at low [^{3}H]RTX concentrations did not allow the determination of the affinity of the vanilloid receptor in membranes preincubated with the sulfhydryl-reactive agents, it was clear that the B_{max} values had been markedly reduced both by DTNB and Cd^{2+} at these concentrations suggesting either a noncompetitive or, alternatively, a mixed inhibitory mechanism (not shown).

Discussion

Capsaicin is widely used as a neuropharmacological probe to dissect a fundamental subdivision of primary afferent neurons, that are activated by noxious chemical, mechanical, and thermal stimuli and are involved in the effector regulation of the tissues they innervate

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Exhibit D

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Gingerols: a novel class of vanilloid receptor (VR1) agonists

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1 Gingerols, the pungent constituents of ginger, were synthesized and assessed as agonists of the capsaicin-activated VR1 (vanilloid) receptor.

2 [6]-Gingerol and [8]-gingerol evoked capsaicin-like intracellular Ca^{2+} transients and ion currents in cultured DRG neurones. These effects of gingerols were blocked by capsazepine, the VR1 receptor antagonist.

3 The potency of gingerols increased with increasing size of the side chain and with the overall hydrophobicity in the series.

4 We conclude that gingerols represent a novel class of naturally occurring VR1 receptor agonists that may contribute to the medicinal properties of ginger, which have been known for centuries. The gingerol structure may be used as a template for the development of drugs acting as moderately potent activators of the VR1 receptor.

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Keywords: Ginger; gingerols; DRG neurones; VR1 receptor; intracellular calcium; plasma membrane currents

Abbreviations: CAP, capsaicin; CBI, cannabinoid receptor; CPZ, capsazepine; DRG, dorsal root ganglion neurones; [6]-G, [6]-gingerol; [8]-G, [8]-gingerol; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; $[Ca^{2+}]_i$, intracellular calcium; NGF, nerve growth factor; VR1, vanilloid receptor subtype-1; VOCC, voltage-operated Ca^{2+} channels

Introduction

Ginger (*Zingiber officinale*) has been used extensively for more than 2500 years in China for conditions including headaches, nausea and colds (Grant & Lutz, 2000) and in Ayurvedic (Sharma & Clark, 1998) and Western herbal medicine practice for the treatment of arthritis, rheumatological conditions and muscular discomfort (Blumenthal & Werner, 1998). Its use in inflammatory conditions is consistent with anti-inflammatory activities of its components *in vitro* (Kiuchi *et al.*, 1982; Mascolo *et al.*, 1989). The moderate pungency of ginger has been attributed to the mixture of gingerol derivatives in the oleoresin fraction of processed ginger (Mustafa *et al.*, 1993). Gingerols possess the vanillyl moiety (Figure 1, region A), which is considered important for activation of the VR1 receptor expressed in nociceptive sensory neurones (Walpole *et al.*, 1993a). Recently it was found that molecules lacking the vanillyl structure also activate the VR1 receptor in DRG neurones. These molecules include *N*-arachidonoyl-dopamine and its 3-*O*-methyl analogue (Huang *et al.*, 2002), anandamide, an endogenous ligand of the neuronal cannabinoid receptor (CB1) (Smart & Jerman, 2000; Smart *et al.*, 2000; Zygmunt *et al.*, 1999), lipoxygenase metabolites (Hwang *et al.*, 2000; Craib *et al.*, 2001; Piomelli, 2001), and the naturally occurring sesquiterpene dialdehydes (Szallasi *et al.*, 1998). The VR1 receptor has recently been cloned and suggested to integrate chemical and thermal nociceptive stimuli (Tominaga *et al.*, 1998; Caterina *et al.*, 1997; 2000). Therefore, direct activation/deactivation of the VR1 receptor at the site where pain is generated during inflammation and other painful conditions provides a new strategy for the development of a new class of peripheral analgesics devoid of the well characterized side effects of currently available analgesics (Kress & Zeilhofer, 1999; Roufogalis & Dedov, 1999). Furthermore, VR1-expressing neurones have recently been found throughout the whole neuroaxis (Mezey *et al.*, 2000), opening up a new and so far unexplored area of VR1-related drug development. We report here for the first time to our knowledge that the pungent principle of ginger, [6]-gingerol and [8]-gingerol, activate the VR1 receptor in capsaicin-sensitive neurones and that activation is blocked by the VR1 antagonist, capsazepine.

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Methods

Materials

Fura-2 and Fluo-4 were obtained from Molecular Probes Inc. (Eugene, U.S.A.), DMEM and Neurobasal medium were from GIBCO (Gaithersburg, U.S.A.), NGF from ICN Biochemicals (Costa Mesa, U.S.A.). Other agents were obtained from Sigma (St. Louis, U.S.A.). All reagents were of analytical grade. Racemic [6]-gingerol (5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)decan-3-one) and [8]-gingerol (5-hydroxy-1-(4-hydroxy-3-methoxyphenyl) dodecan-3-one) were prepared as described in the literature (Denniff *et al.*,

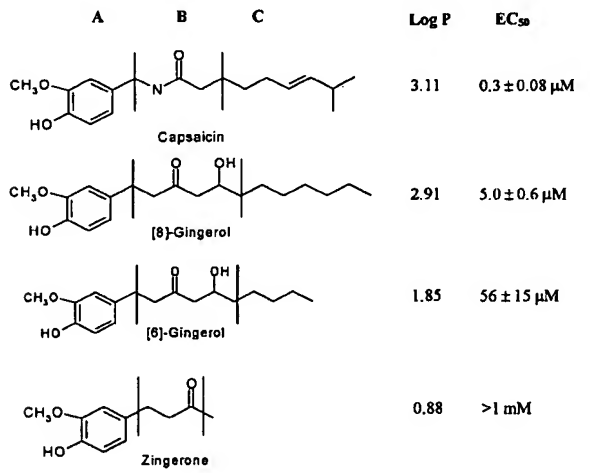

Figure 1 Comparison of capsaicin, [6]-gingerol, [8]-gingerol and zingerone. The molecules are divided into 'A', 'B' and 'C' regions, as described for capsaicin by Walpole *et al.* (1993a–c). Log P values were calculated using Molecular Modeling Pro software (ChemSW, version 3.23). EC_{50} values for plasma membrane current flow were determined as described in the Results.

1981). Log *P* values of the compounds were calculated using Molecular Modeling Pro software (ChemSW, version 3.23).

Preparation of rat DRG neurones

This study was approved and carried out in accordance with the guidelines of the Animal Ethics Committee of The University of Sydney (AEC approval No: L24/11-99/2/3047). Isolated DRG from neonatal (3–5-day-old) rats were incubated in Hanks CMF saline with 0.05% collagenase and 0.25% trypsin for 25 min at 37°C. Neurones were obtained by trituration of ganglia with fire polished Pasteur pipettes of decreasing diameters and afterwards the cellular suspension was washed twice in DMEM medium supplemented with 10% foetal calf serum (FCS) and 2 mM glutamine. Freshly isolated neurones were plated onto collagen-coated coverslips (for intracellular Ca^{2+} assay) or on plastic petri dishes (for electrophysiological studies). Neurones were cultured in: (i) Neurobasal medium with B27 supplement, 50 ng ml^{-1} 2.5 S nerve growth factor (NGF) for intracellular Ca^{2+} assay; (ii) DMEM medium supplied with 10% foetal calf serum for electrophysiological studies. All media contained 2 mM glutamine, 100 u ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. All cultures of neurones were kept at 37°C with 5% CO_2 overnight.

Measurement of intracellular Ca^{2+} transients in rat DRG neurones using Fura-2 AM

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) was measured as described previously (Dedov & Roufogalis, 1998; 2000). Briefly, DRG neurones on coverslips were incubated with 5 μM Fura-2 AM for 30 min at 37°C. The coverslips were then mounted on a chamber attached to a rapid sample perfusion system. Single cell recordings were made on the stage of a Nikon Diaphot inverted microscope fitted with a Nikon 40 × Fluo (NA 0.85) DL Ph3 or 40 × Fluo (NA 1.3) oil objective. $[Ca^{2+}]_i$ was calculated from dual excitation wavelength (340/380 nm) fluorescence measurements following an intracellular calibration procedure by the Grynkiewicz equation (Grynkiewicz *et al.*, 1985; Kao, 1994) using MCID M2/M4 v.3.0 software (Imaging Res. Inc. St. Catharines, Ont., Canada). Cells were continuously perfused with a solution consisting of (in mM) NaCl 140, $CaCl_2$ 2, KCl 5, HEPES 20, glucose 10, pH 7.4. The cytoplasmic localization of Fura-2 was confirmed with the Mn^{2+} quenching technique (Dedov & Roufogalis, 1998). All experiments were performed at room temperature (20–22°C). A minimum of three separate cultures of 10–20 neurones was used for each experiment.

Measurement of intracellular Ca^{2+} transients in rat DRG neurones using Fluo-4 probe

Fluo-4 was used in a TCS SP2 Leica imaging system to measure intracellular Ca^{2+} transients in DRG neurones in capsazepine antagonism experiments with lower concentrations of capsaicin (100 nM). DRG cultures on coverslips were incubated with 10 μM Fluo-4 for 15 min at 37°C and mounted onto the Leica microscope using a laminar flow chamber, as described in the Fura-2 measurements above. Fluorescence changes of Fluo-4 were recorded every 2 s on the Leica system fitted with HC × PL APO 63x/1.20 W CORR water-objective. The perfusion system was similar to that described for Fura-2 measurements. In each experiment neurones were first exposed to a low dose of capsaicin (100 nM) for 5–10 s, followed by addition of [6]-gingerol (10 μM) or [8]-gingerol (10 μM), then KCl (50 mM) was added. A 3-min wash was employed between each addition of the drugs. This low concentration and short duration capsaicin exposure regime was designed to identify capsaicin-sensitive neurones and to minimize desensitization of neurones upon repeated applications of the drugs. In the capsazepine antagonism experiment neurones were first exposed to capsaicin (100 nM) followed by a 3-min wash, then capsazepine, gingerol and capsaicin were added in sequence. Neurones were finally washed with incubation buffer then KCl (50 mM) was added. The Ca^{2+}-dependent fluorescence changes were calibrated at the end of experiments by adding ionomycin (10 μM) in the presence of 20 mM extracellular Ca^{2+} to obtain F_{max} (saturated levels of fluorescence), followed by cell lysis in the presence of 50 μM digitonin to obtain F_{min} (background fluorescence). The Ca^{2+} transients were represented as a ratio F/F_{max} *versus* time. All experiments were performed at 20–22°C.

Electrophysiological studies

DRG neurones were prepared as described in Fura-2 AM experiments. Voltage-clamp recordings were made with whole-cell patch-clamp techniques using an AxoPatch 1D patch-clamp amplifier (Axon Instruments, Foster City, CA, U.S.A.). Micropipettes were pulled from borosilicate glass capillary tubing (7052 Glass, A-M Systems, Everett, WA) and had d.c. resistance of 0.8–2.0 MΩ. To record macroscopic currents, micropipettes were filled with a solution of the following composition (in mM): CsCl 130, NaCl 5, N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) 10, EGTA 10, $CaCl_2$ 2, MgATP 5 and NaGTP 0.3, with the pH adjusted to 7.3 with CsOH, osmolarity approximately 285 mosml. The external bathing solution contained (in mM):

NaCl 140, $MgCl_2$ 1.5, $CaCl_2$ 2.5, KCl 2.5, glucose 10, HEPES 10, with the pH adjusted to 7.3 with NaOH and osmolarity approximately 330 mosml. Data were recorded at room temperature (20–22°C). Concentration response data was collected at a holding current of +40 mV, reversal potentials were determined by means of a ramp I/V, from +40 mV to −60 mV, as shown in Figure 4A. The liquid junction potential between internal and external solutions was approximately 4.5 mV, and all data were compensated for this value. Stimulation and recording were both controlled by a pClamp 5.5 data acquisition system (Axon Instruments). Data were filtered at 200 Hz (4 pole low-pass Bessel filter) and digital sampling rates were between 1 and 5 kHz, depending on the voltage protocol length. Capacitive currents were manually nulled and series resistance compensation of at least 80% was used for all cells. Concentration-response relationships were obtained by exposing cells to increasing concentrations of test compound at approximately 2-min intervals. Compounds were perfused until the current reached equilibrium, and the currents were compared to those caused by application of a maximally effective concentration of capsaicin (10 μM) applied at the end of the experiment. Application of drugs to the cells was carried out *via* a series of flow pipes in a similar procedure as described by Piper *et al.* (1999). Cells were voltage clamped at +40 mV to reduce calcium entry through VR1. Desensitization at +40 mV was negligible even after 2-min applications of capsaicin and this was much less than at −60 mV.

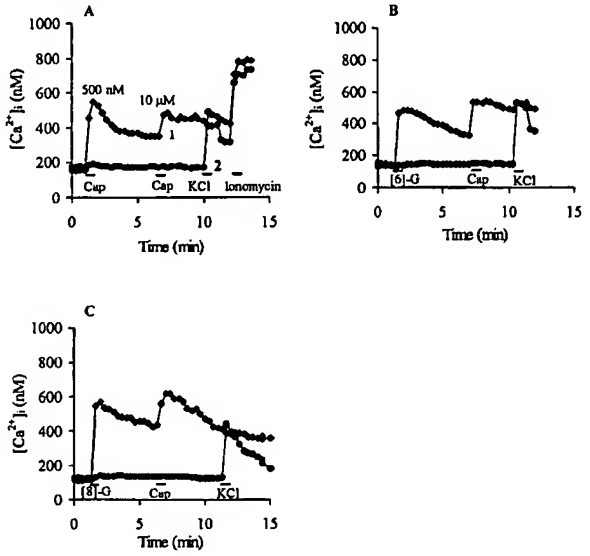

Figure 2 $[Ca^{2+}]_i$ transients in Fura-2 loaded DRG neurones evoked by capsaicin and constituents of ginger. Drugs were applied for 1 min where indicated by bars, following extensive wash out with physiological solutions. Successive drug applications were: (A) 500 nM capsaicin (Cap), 10 μM capsaicin, 50 mM KCl, 2 μM ionomycin; (B) 10 μM [6]-gingerol ([6]-G), 10 μM capsaicin, 50 mM KCl; (C) 10 μM [8]-gingerol ([8]-G), 10 μM capsaicin, 50 mM KCl. Data from Fura-2 loaded DRG neurones typical of 10–20 cells from at least three separate cultures represent capsaicin and gingerol evoked $[Ca^{2+}]_i$ transients (trace 1) and capsaicin and gingerol-insensitive $[Ca^{2+}]_i$ transients (trace 2).

Results

Measurement of Ca^{2+} transients in Fura-2 loaded dorsal root ganglion cells

To determine whether gingerols activate the VR1 receptor, we have synthesized two gingerols representative of those present in natural ginger ([6]-gingerol and [8]-gingerol) and a gingerol degradation product (zingerone) devoid of the hydrophobic side chain (Figure 1, region C). Despite the obvious similarity of gingerol and capsaicin structures, the activity of gingerols on the VR1 receptor has yet to be reported to our knowledge.

Application of capsaicin to cultured DRG neurones loaded with Fura-2 evoked $[Ca^{2+}]_i$ transients in the capsaicin-sensitive population of DRG neurones (Figure 2A, trace 1). These $[Ca^{2+}]_i$ transients are due to influx of extracellular Ca^{2+} upon activation of the VR1 receptor and serve as characteristic markers of capsaicin-sensitive DRG neurones (Caterina *et al.*, 1997; Cholewinski *et al.*, 1993; Dedov & Roufogalis, 1998; 2000). A capsaicin-insensitive population of DRG neurones did not respond to capsaicin, but responded to the application of 50 mM KCl (Figure 2A, trace 2), which evoked extracellular Ca^{2+} influx *via* voltage-operated Ca^{2+} channels (VOCC) in all DRG neurones (Kostyuk & Verkhratsky, 1995). Application of capsaicin inhibited responses to the subsequent application of KCl in capsaicin-sensitive DRG neurones (Figure 2A–C), likely due to inhibition of VOCCs by capsaicin (Kopanitsa *et al.*, 1995). KCl-insensitive cells were considered to be non-neuronal cells. Neither capsaicin nor gingerols evoke $[Ca^{2+}]_i$ elevation in these cells (results not shown). A further increase in $[Ca^{2+}]_i$ upon application of 2 μM ionomycin following the agonist application indicated that these responses were not limited by saturation of the Fura-2 signal (Figure 2A).

Typical traces showing that [6]-gingerol (10 μM) and [8]-gingerol (10 μM) evoked $[Ca^{2+}]_i$ transients exclusively in the capsaicin-sensitive DRG neurones are shown in Figure 2B,C. In all cases (over 180 neurones analysed in 12 separate experiments) cells that responded to gingerols also responded to subsequent application of 10 μM capsaicin and *vice versa*, whereas capsaicin-insensitive cells responded neither to gingerol nor to capsaicin (Figure 2B,C).

Gingerol evoked $[Ca^{2+}]_i$ transients in capsaicin-sensitive neurones was also demonstrated in rat DRG neurones loaded with Fluo-4, as shown in Figure 3A,C. Under the experimental conditions described, gingerols induced a rapid rise in intracellular calcium, similar to that produced by capsaicin. The continued rise in $[Ca^{2+}]_i$ transients (F/F_{max} ratios) after cessation of application of drug has been observed in previous studies (Cholewinski *et al.*, 1993) where there is a lag phase between activation and inactivation of the VR-1 receptors in response to capsaicin. The continued rise in $[Ca^{2+}]_i$ after removal of drug may result from the difficulty of washing out drugs from the membrane localization, Ca^{2+}-dependent signalling processes or Ca^{2+} entry *via* VOCC's (Kostyuk & Verkhratsky, 1995). It is worth noting that repeated applications of capsaicin at 100 nM showed little or no desensitization of neurones (over 80 neurons in 24 separate experiments) under conditions described in these experiments (results not shown). The activation of DRG neurones by gingerols was again only shown to occur in neurones that are sensitive to capsaicin. This agonistic

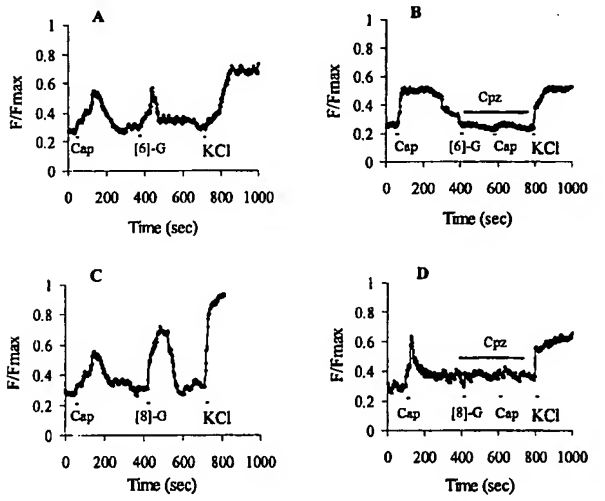

Figure 3 Effect of pre-treatment of DRG neurones with capsaicin and capsazepine on gingerol $[Ca^{2+}]_i$ transient responses. $[Ca^{2+}]_i$ transients are represented as an increase in fluorescence ratio (F/F_{max}; basal amplitude = 0.3 ± 0.09) of Fluo-4 loaded DRG neurones (typical of 20–30 cells from at least three separate cultures) evoked by capsaicin, [6]-gingerol and [8]-gingerol. Drugs were applied for 5–10 s, as shown by bars in the Figure, following a 3-min wash out with physiological solutions between additions. Capsazepine applications are shown as indicated by bars. Successive drug applications were: (A) 100 nM capsaicin (Cap), 10 μM [6]-gingerol ([6]-G) and 50 mM KCl; (B) 100 nM capsaicin, 10 μM capsazepine (Cpz), 10 μM [6]-gingerol, 100 nM capsaicin and 50 mM KCl; (C) 100 nM capsaicin, 10 μM [8]-gingerol ([8]-G) and 50 mM KCl; (D) 100 nM capsaicin, 10 μM capsazepine, 10 μM [8]-gingerol, 100 nM capsaicin and 50 mM KCl.

activity was blocked by capsazepine, a capsaicin antagonist, as shown in Figure 3B,D. Addition of capsaicin (100 nM) to neurones previously treated with capsazepine showed no $[Ca^{2+}]_i$ response.

These data suggest that gingerols exhibit agonist activity towards the VR1 receptor in rat DRG neurones similar to that of capsaicin. However, the absolute efficacy of gingerols at the VR1 receptor could not be determined from this study as gingerol-induced elevations of $[Ca^{2+}]_i$ may represent both calcium entry through VR1 receptors and calcium entry resulting from membrane depolarization and opening of VOCCs. Moreover, primary DRG neurons in culture differ in holding potential and $[Ca^{2+}]_i$ handling capacities (Kostyuk & Verkhratsky, 1995). This makes it impossible to study relative efficacy of gingerols by this technique and Figures 2 and 3 represent gingerol effects only qualitatively.

The patch-clamp technique, however, allows normalization of plasma membrane holding potentials and, therefore, comparative study of drug efficiencies. Hence the plasma membrane currents induced by capsaicin and gingerols in the presence and absence of capsazepine were also examined, as follows.

Measurement of plasma membrane currents

Plasma membrane currents induced by capsaicin in DRG neurones were outward at positive membrane potentials and inward at negative potentials, and reversed polarity at -6 ± 1 mV ($n=9$), with an EC_{50} of 0.3 ± 0.08 μM (Figure 4). Both [6]-gingerol and [8]-gingerol induced increases in the plasma membrane conductance exclusively in capsaicin-sensitive DRG neurones, as in all cells examined neurones that did not respond to capsaicin did not respond to the application of gingerols (results not shown). Currents evoked by gingerols mimicked those evoked by capsaicin, with reversal potential of -6 ± 2 mV ($n=6$) for [8]-gingerol and -5 ± 1 mV ($n=7$) for [6]-gingerol (Figure 4A). Concentration-response curves were determined by application of agonist to neurones at increasing concentrations at a voltage clamped at +40 mV (to minimize Ca^{2+} entry and desensitisation as explained in experimental section), and the currents were normalized to those produced by 10 μM capsaicin in each cell. [8]-gingerol (EC_{50} 5.0 ± 0.6 μM) was more potent than [6]-gingerol (EC_{50} 56 ± 15 μM) in inducing plasma membrane conductance. In contrast zingerone, even at high concentration (1 mM), produced a current only approximately 10% of the maximum current induced by capsaicin. [8]-Gingerol and [6]-gingerol produced maximum currents similar in magnitude to that of capsaicin (10 μM) (Figure 4B). Currents evoked by 1 μM [8]-gingerol, 10 μM [6]-gingerol and 1 μM capsaicin were almost completely blocked by pre-application of 10 μM capsazepine (Table 1), which corresponds to a >99% reduction in the current, if considered on currents normalized in each cell. All the neurones where the capsazepine block of current was tested had been previously shown to be sensitive to capsaicin or one of the gingerols. It should be noted that under the

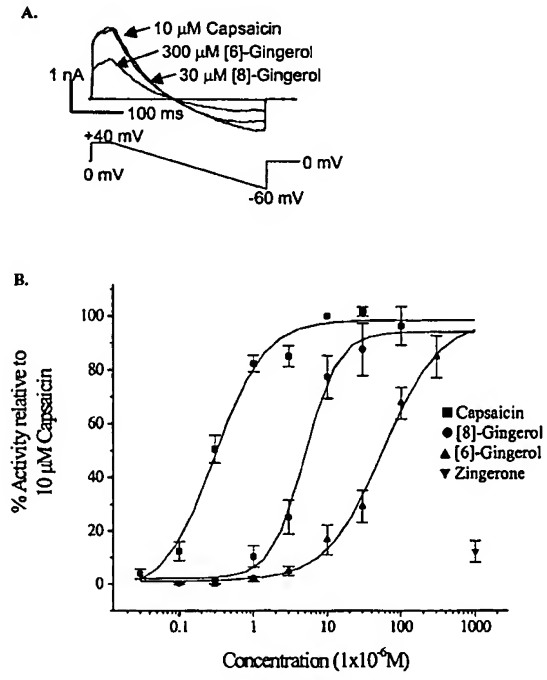

Figure 4 Ion currents evoked by capsaicin and gingerols in whole-cell patch clamped DRG neurones. (A) Voltage ramp was applied to patch-clamped DRG neurons in the presence of 10 μM capsaicin, 30 μM [8]-gingerol and 300 μM [6]-gingerol. Note the same I/V characteristics for all three drugs. (B) Ion current dose response to capsaicin, [8]-gingerol, [6]-gingerol and zingerone calculated as percentage to the amplitude of current evoked by 10 μM capsaicin. Data are presented as mean $\pm$ s.d of at least six neurones per data point.

experimental conditions chosen desensitisation of capsaicin response was minimal. Capsaicin (10 μM) was applied three times, each separated by 3 min, to DRG neurones which were clamped at voltage of +40 mV to minimize calcium entry. The second application produced a current 103±3% of the first, the third application produced a current 106±6% of the first ($n=6$).

Direct measurement of channel currents of the agonists at a ligand-gated ion channel reflects the efficacy of the agonists. [8]-Gingerol gave a current at the highest concentration tested of 96±7 % the current caused by application of a maximal concentration of capsaicin. This indicates that the efficacy of the two compounds at VR1 is very similar and that [8]-gingerol is probably a full agonist. These data indicate that the gingerols activate capsaicin-like membrane conductance and may be considered as full agonists of the VR1 receptor.

Table 1 The effect of capsaicin and gingerols on plasma membrane current of capsaicin sensitive DRG neurones in the presence and absence of capsazepine

	Current (pA)	
Compound	*Control*	*Capsazepine* (10 μM)
Capsaicin (1 μM)	2496±1148 ($n=5$)	70±56 ($n=5$)
[6]-Gingerol (30–100 μM)	2595±479 ($n=6$)	42±39 ($n=6$)
[8]-Gingerol (10 μM)	1449±226 ($n=5$)	0±2 ($n=5$)

Data presented is the mean (±s.d.) of at least five independent experiments.

Discussion

In this study we have shown for the first time that gingerol constituents of ginger are relatively potent and efficacious agonists of the VR1 receptor. The activity of gingerols depends on the size of the side chain, which also determines their hydrophobicity. Increasing the number of carbons from 6 to 8 and the hydrophobicity index from 1.90 to 2.88 in the transition from [6]-gingerol to [8]-gingerol coincided with about a 10 fold increase in potency in inducing plasma membrane currents (Figure 4B). Absence of the side chain in zingerone, a ketone product of gingerol degradation, dramatically reduced its activity towards the VR1 receptor by a magnitude of at least 100 fold compared to that of [6]-gingerol (Figure 4B). This finding was further supported by a study by Liu & Simon (1996) which showed that zingerone weakly activated inward current with a threshold concentration of 1 mM, which was approximately 10,000 fold less potent than capsaicin (threshold concentration of 0.1 μM) in inducing inward current in rat trigeminal ganglion cells (Liu & Simon, 1996).

Structure activity studies of capsaicin-like compounds have previously divided the capsaicin molecule into three regions, as shown in Figure 1; the aromatic 'A' region (Walpole *et al.*, 1993a), the amide bond 'B' region (Walpole *et al.*, 1993b), and the hydrophobic side-chain 'C' region (Walpole *et al.*, 1993c). In this study [8]-gingerol exhibited lower potency than capsaicin in activation of VR1 receptor, despite both having approximately the same number of carbons in the side chain. The presence of the hydroxyl moiety at C_5 in the 'B' region of [8]-gingerol could be significant in altering the potency of activation of the VR1 receptor. Substitution of an amide function in capsaicin appears to increase its hydrophobicity (Log $P=3.11$), and this coincides with a 20 fold higher potency of capsaicin in comparison to [8]-gingerol.

Discovery of gingerols as potent VR1 receptor agonists serves to explain the traditional and recent use of ginger for pain relief in rheumatic and inflammatory conditions (Blumenthal & Werner, 1998; Srivastava & Mustafa, 1992). Moreover, the presence of VR1 receptors throughout the brainstem (Mezey *et al.*, 2000), where the nausea centre is located, may conceivably be associated in part with the common use of ginger as antiemetic medicine (Ernst & Pittler, 2000; Mustafa *et al.*, 1993). The therapeutic value of capsaicin as a medicinal agent is limited by its high pungency and neurotoxicity (Wood, 1993). Topical application of a capsaicin-containing lotion has resulted in the loss of epidermal nerve fibres (Nolano *et al.*, 1999). It appears that the toxic effects of capsaicin are associated with VR1 receptor mediated extracellular Ca^{2+} influx (Chard *et al.*, 1995) and release of intracellular Ca^{2+} from internal stores in the endoplasmic reticulum (Olah *et al.*, 2001), as well as the effect of capsaicin on mitochondria in DRG neurones (Dedov & Roufogalis, 2000). Therefore, further exploring the functional groups on the side chain of gingerol structures would allow for development of more desirable pharmacological compounds acting *via* the VR1 receptor for controlling pain.

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Full Paper

Modifications of Capsaicin-Sensitive Neurons in Isolated Guinea Pig Ileum by [6]-Gingerol and Lafutidine

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Abstract. A segment of guinea pig ileum was used to confirm the hypothesis that [6]-gingerol and lafutidine interact with capsaicin-sensitive neurons. Addition of 30 and 100 μM [6]-gingerol (a pungent constituent of ginger) induced contraction of the ileum immediately. Like capsaicin, [6]-gingerol-induced contraction was inhibited by antagonists of the vanilloid receptor (capsazepine and ruthenium red), tetrodotoxin, and atropine. Treatment with [6]-gingerol up to 0.3 μM, which alone had no effect, enhanced 3 μM capsaicin-induced contraction, but greater than 3 μM [6]-gingerol significantly inhibited capsaicin-induced contraction. Treatment with lafutidine (a new type of antagonist of the histamine H_2 receptor), which was suggested to interact with capsaicin-sensitive neurons in vivo, also showed both stimulatory and inhibitory effects on capsaicin-induced contraction depending on the concentrations. Lafutidine alone had no effect. The enhanced contraction induced by capsaicin in the [6]-gingerol- or lafutidine-treated ileum was also inhibited by antagonists of the vanilloid receptor, tetrodotoxin, and atropine. Capsaicin and [6]-gingerol, but not lafutidine, at 30 μM stimulated [^{3}H]choline release from the prelabeled slices of the ileum. These findings suggest that [6]-gingerol and lafutidine act on capsaicin-sensitive cholinergic neurons and modulate the contraction in isolated guinea pig ileum.

Keywords: [6]-gingerol, lafutidine, vanilloid receptor, enteric nervous system, guinea pig ileum

Introduction

Capsaicin, the major pungent constituent of hot peppers of the plant genus *Capsicum*, excites a subset of primary sensory neurons with somata in dorsal root ganglia or trigeminal ganglia (1, 2). A functional receptor termed the vanilloid receptor 1 (VR1), which is activated not only by vanilloids such as capsaicin but also by noxious heat and low pH, has been cloned (3). In general, capsaicin-sensitive neurons transmit noxious information perceived as pain or itching to the central nervous system. Activation of capsaicin-sensitive neurons in the peripheral tissues also evoked various responses (1, 4, 5). In the ileum, activation of primary afferent neurons by capsaicin causes release of transmitter substances from the neurons, which in turn activate myenteric cholinergic neurons and induce contraction of the ileum by activation of muscarinic acetylcholine (ACh) receptors (5 – 9).

Ginger is widely used as a spice throughout the world, and the rhizome of ginger has been used in traditional oriental medicine to ameliorate such symptoms as gastrointestinal discomforts, inflammation, and rheumatic disorders for centuries in East Asia. Gingerols including [6]- and [8]-gingerol are the pungent constituents of ginger, *Zingiber officinale* ROSCOE (a traditional Sino-Japanese medicine, a *Kampo* medicine). Gingerols exhibited various pharmacological effects in cardiovascular systems (10, 11) and showed positive inotropic effects on isolated atria and cardiac muscles (12 – 14). Zingerone, another pungent constituent of ginger, has been shown to have similar pharmacological effects as capsaicin, and thus it was suggested that capsaicin and zingerone could activate the same receptor and/or a common pathway in trigeminal ganglion neurons (15 – 17). However, there is no evidence

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concerning the effects of gingerols on the enteric nervous system. In the present study, we investigated whether [6]-gingerol activates capsaicin-sensitive neurons in the guinea pig ileum.

Lafutidine is a new type of antagonist of histamine H_2 receptors (18, 19). Since the protective effects of lafutidine against gastric ulcers were totally abolished by loss of function of capsaicin-sensitive sensory neurons in rats in vivo (19 – 22), it is suggested that the capsaicin-sensitive pathways are involved in gastro-protection induced by lafutidine. To confirm the hypothesis that [6]-gingerol and lafutidine interact with capsaicin-sensitive neurons, we examined the effects of two agents on the capsaicin-induced contraction and ACh release in the isolated ileum preparation from guinea pigs.

Materials and Methods

Animals and materials

Male, albino Dunkin-Hartley guinea pigs (300 – 400 g) were purchased from Takasugi Lab. Animals Co., Ltd. (Saitama). The animals were housed under controlled environmental conditions (temperature 24 ± 2°C and light between 7:00 a.m. and 7:00 p.m.) and fed commercial MF chows (Oriental Yeast Co., Ltd., Tokyo). The animals were fasted overnight before each experiment with free access to water. Animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

[Methyl-^{3}H]choline chloride (79 Ci/mmol (2.92 TBq /mmol)) was purchased from Amersham (Buckinghamshire, UK). Capsaicin and ACh chloride were obtained from Wako (Osaka). Capsazepine was from RBI (Natic, MA, USA). Ruthenium red, famotidine, and ionomycin were purchased from Sigma (St. Louis, MO, USA). Hemicholinium-3 was obtained from Sigma-Aldrich (Steinheim, Germany). [6]-Gingerol and atropine sulfate were obtained from Nacalai Tesque (Kyoto). Hexamethonium chloride and tetrodotoxin were obtained from Tokyo-Kasei (Tokyo) and Sankyo (Tokyo), respectively. CP96345 ((2*S*,3*S*)-*cis*-2-(diphenylmethyl)-*N*-[(2-methoxyphenyl)-methyl]-1-azabicyclo[2.2.2]octan-3-amine) was from Pfizer, Inc. (Groton, CT, USA). Substance P and lafutidine were obtained from Peptide Institute, Inc. (Osaka) and Taiho Pharmaceutical Co., Ltd. (Tokyo), respectively. Capsaicin and [6]-gingerol were dissolved in a minimum of ethanol, and capsazepine and lafutidine were dissolved in a minimum of dimethyl sulfoxide. These agents were diluted with the indicated buffer when used, and the final concentration of ethanol or dimethyl sulfoxide in the assay was less than 0.5% (v/v). The pHs of the vehicles containing ethanol and/or dimethyl sulfoxide and the agents used in experiments were pH 6.5 – 7.4, and the vehicle (containing 0.5% ethanol or dimethyl sulfoxide) had no effect on the contraction and the [^{3}H]choline release in the guinea pig ileum.

Isolated ileum preparation and measurement of contraction

The ileum preparation and measurement of contraction was done as described previously (23). Briefly, the whole segments of guinea pig ileum were removed in Krebs-Henseleit buffer (112.0 mM NaCl, 5.9 mM KCl, 2.0 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25.0 mM $NaHCO_3$, 11.5 mM glucose; pH 7.4). The segment of ileum was set up under a 1-g load in a 5-ml organ bath containing the buffer. The bath was maintained at 32°C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2. Contraction was recorded using an isotonic transducer (Type 45347; NEC San-ei, Tokyo). At the start of each experiment, a maximal response to 3 μM ACh was obtained in each tissue to estimate the effects of the tested agents. To investigate the susceptibility of capsaicin- and [6]-gingerol-induced contraction to desensitization, segments of the ileum were incubated for 7 min with the indicated concentrations of capsaicin or [6]-gingerol, then washed more than 3 times with the stimulant-free buffer, and incubated with the indicated agents to measure contraction for the second time. The interval between the first exposure to the agents and the second exposure to the agents was about 30 min. Since the second responses of capsaicin and [6]-gingerol were desensitized easily in the same preparation as shown in Results, the concentration-response curves of capsaicin and [6]-gingerol were obtained in another ileal segment isolated from the same animal. In some experiments, segments of the ileum were incubated for 7 min with the antagonists or inhibitors, and then the response induced by contractive agents was measured in the presence of the respective antagonists or inhibitors. The antagonists or inhibitors were used at concentrations equal to or 2 times higher than those used in previous reports (5, 24 – 26). The maximal contraction induced by ACh was obtained at the concentration of 3 μM, and each response was expressed as a percentage of the contraction due to 3 μM ACh (% of ACh contraction). The addition of capsaicin or [6]-gingerol caused somewhat irregular responses, and thus the value of the peak contraction was used for the calculation.

[^{3}H]Choline release from the prelabeled slices of the guinea pig ileum

The measurement of [^{3}H]choline and [^{3}H]ACh releases

from the prelabeled slices of the guinea pig ileum was carried out as follows: The mucosal component in the segments of guinea pig ileum was denuded by gently rubbing with wet-cotton. The segments were further cross-chopped to slices (400 × 400 μm) by hand, followed by filtration through a mesh (300 × 300 μm). The slices were washed twice with a modified Tyrode HEPES buffer (137 mM NaCl, 5 mM KCl, 5 mM glucose, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 20 mM HEPES; pH 7.4) followed by centrifugation at 4°C (100 × g, 30 s). Several investigators reported that the tissues in a superfusion system were subjected to electrical stimulation in order to reduce endogenous acetylcholine, and then the acetylcholine stores were labeled with [^{3}H]choline (27, 28). In the present study, the slices were incubated with 5 μM ionomycin for 30 min, and the washed slices were incubated for 60 min with [^{3}H]choline (2.5 μCi/ml) at 37°C. [^{3}H]Choline incorporated into the cholinergic nerve terminals is synthesized to [^{3}H]ACh. The labeled slices were washed twice and suspended in the buffer. The labeled slices (300 – 500 μg protein) were incubated with the indicated agents for 10 min at 37°C. The assay mixture was further supplemented with 10 μM hemicholinium-3 to prevent the uptake of choline formed from [^{3}H]ACh. The total volume was 300 μl and the reaction was terminated by the addition of 500 μl of ice-cold, Ca^{2+}-free, Mg^{2+}-free Tyrode buffer containing 5 mM EGTA and 5 mM EDTA followed by centrifugation (2,000 × g, 30 s) at 4°C. The ^{3}H content (the sum of [^{3}H]choline and [^{3}H]ACh) in the supernatant was estimated by liquid scintillation spectrometry. The values represented in the [^{3}H]choline release are the ratio of released ^{3}H content to the total incorporation of [^{3}H]choline into the slices.

Statistical analyses

Values are presented as means ± S.E.M. for greater than 3 independent experiments. The number of experiments (n) refers to the number of experimental animals used. The statistical significance of differences between two groups was assessed using the two tailed Student's t-test. Multiple comparisons against a single control group were made by one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test. $P<0.05$ was considered statistically significant.

Results

Contraction induced by [6]-gingerol in guinea pig ileum

First, we confirmed 3 μM capsaicin-induced contraction in the whole segments of guinea pig ileum (Fig. 1, trace a). As described previously (5 – 9), the contraction induced by capsaicin was concentration-dependent. The maximal contraction was produced by the concentrations greater than 3 μM capsaicin, and that was about 20 – 30% of the contraction induced by 3 μM ACh (% of ACh contraction) in the present study. The effect of capsaicin was susceptible to desensitization, as in previous reports (5, 8). The segments of ileum were incubated with 3 μM capsaicin for 7 min and then thoroughly washed with the capsaicin-free buffer and used as capsaicin-treated ileum. Contraction of the capsaicin-treated ileum induced by the second exposure to 3 μM capsaicin was 1.2 ± 0.9% (n = 5), which was significantly lower than the control contraction (23.4 ± 4.4%, n = 8).

[6]-Gingerol showed no effect at lower concentrations (up to 3 μM), and it induced contraction in the ileum at higher concentrations greater than 30 μM (Fig. 1, traces b and c; Fig. 2A). The [6]-gingerol-induced contraction was susceptible to desensitization; the contraction induced by the first challenge of 30 μM [6]-gingerol was 13.3 ± 2.8% (n = 6), but the second challenge hardly elicited the contraction in the [6]-gingerol-treated ileum (2.0 ± 0.9%, n = 3, $P<0.01$). Capsazepine is a competitive antagonist, and ruthenium red is a functional and non-competitive antagonist of the vanilloid receptor (1, 2). In guinea pig ileum, both the antagonists at 10 μM inhibited not only 3 μM capsaicin- but also 30 μM [6]-gingerol-induced contraction markedly (Table 1). These antagonists for the vanilloid receptor did not inhibit the submaximal and maximal contractions induced by 100 nM and 3 μM ACh, respectively (n = 4). The contractions induced by 3 μM capsaicin and 30 μM [6]-gingerol in the control ileum were markedly inhibited by atropine (1 μM, an antagonist of muscarinic ACh receptor) and by tetrodotoxin (100 nM, an inhibitor of voltage-dependent Na^+ channels) treatment.

Treatment of the segment of guinea pig ileum for 40 min with 20 μM indomethacin (an inhibitor of cyclooxygenases) did not modify the contractions induced by 100 μM [6]-gingerol and by 3 μM capsaicin (Table 2). Treatment with hexamethonium (100 μM, an antagonist of nicotinic ACh receptor and thus a ganglionic blocking agent) for 10 min did not show an inhibitory effect on [6]-gingerol- and capsaicin-induced contraction. Treatment with 2 μM CP96345, an antagonist for neurokinin 1 (NK_1) receptors (29), almost completely inhibited the contraction induced by 1 nM substance P, but not that by 100 μM [6]-gingerol and 3 μM capsaicin. The maximal contraction induced by 100 nM substance P was also markedly inhibited in the CP96345-treated ileum: 70 – 80% and 30 – 35% (of ACh response) in the control and the CP96345-treated ileum, respectively.

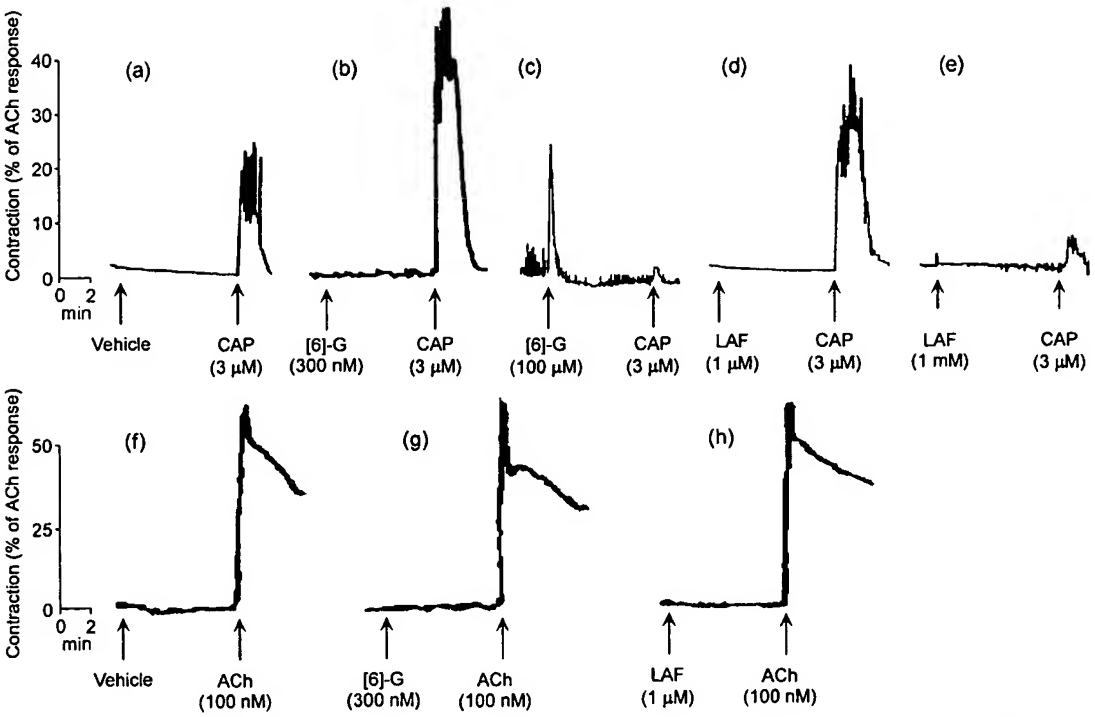

Fig. 1. Modifications of capsaicin-induced contraction by [6]-gingerol and lafutidine. Segments of the guinea pig ileum were incubated with vehicle or the indicated concentrations of capsaicin (CAP), [6]-gingerol ([6]-G), and lafutidine (LAF). At 7 min after the administration, the same segment was incubated with 3 μM capsaicin (traces a – e) or 100 nM ACh (traces f – h) to measure contraction. The recordings are one example of 5 – 7 independent experiments. The peak contraction was expressed as a percentage of the contraction induced by 3 μM ACh.

Dual effects of [6]-gingerol treatment on capsaicin-induced contraction

Treatment with [6]-gingerol at lower concentrations between 3 nM and 0.3 μM, which alone did not induce contractions, enhanced the 3 μM capsaicin-induced contractions; the contraction was significantly enhanced in the 0.3 μM [6]-gingerol-treated ileum (Fig. 1, trace b and Fig. 2A). The enhanced contraction induced by capsaicin in the [6]-gingerol-treated ileum was inhibited by the antagonists of the vanilloid receptor, atropine, and tetrodotoxin (Table 1). In addition, pretreatment with [6]-gingerol at concentrations greater than 3 μM inhibited the contraction induced by the second challenge of 3 μM capsaicin; the contraction was almost completely abolished in the 100 μM [6]-gingerol-treated ileum (Fig. 1, trace c). The submaximal contraction induced by 100 nM ACh (Fig. 1, traces f and g) and the maximal contraction by 3 μM ACh in the 0.3 μM [6]-gingerol-treated ileum were almost the same as those in the control ileum (n = 4). In the 100 μM [6]-gingerol-treated ileum, the response by 100 nM ACh was slightly (20 – 30%) inhibited, and the response by 3 μM ACh was hardly (82.4 ± 5.7%, n = 4, $P = 0.054$) inhibited.

Dual effects of lafutidine treatment on capsaicin-induced contraction

Next, we investigated whether lafutidine regulates capsaicin-induced contraction in isolated guinea pig ileum (Fig. 1, traces d and e). Lafutidine alone did not induce contraction in the ileum at any concentrations (Fig. 2B). Treatment with lafutidine at low concentrations from 0.1 to 1 μM enhanced the contraction induced by 3 μM capsaicin; the contraction was significantly enhanced in the 1 μM lafutidine-treated ileum. The enhanced contraction induced by capsaicin in the 1 μM lafutidine-treated ileum was almost completely eliminated by the antagonists for the vanilloid receptor, atropine, and tetrodotoxin (Table 1). Pretreatment with lafutidine at higher concentrations between 10 μM and 1 mM inhibited the contraction induced by 3 μM capsaicin (Fig. 2B). The capsaicin-induced contraction in the 100 μM lafutidine-treated ileum was significantly smaller than that in the 1 μM lafutidine-treated ileum

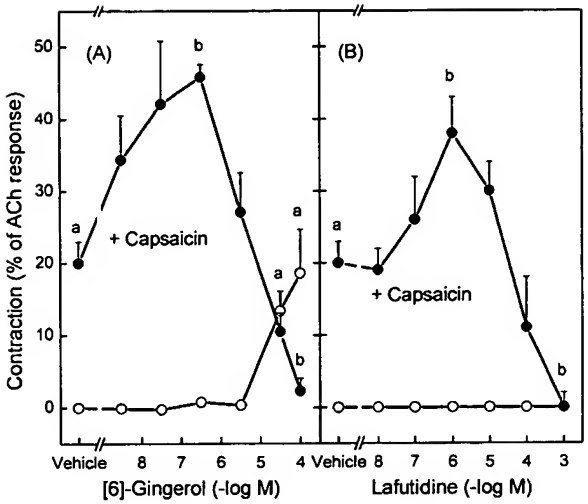

Fig. 2. Concentration dependency of [6]-gingerol or lafutidine. The segments were incubated with the indicated concentrations of [6]-gingerol (Panel A) or lafutidine (Panel B) to measure the first contraction (open circle). At 7 min after the administration of the indicated concentrations of [6]-gingerol or lafutidine, the same segment was incubated with 3 μM capsaicin to measure capsaicin-induced contraction (closed circle). Values are each the mean ± S.E.M. for 4 – 7 independent animals. [a]$P<0.05$, statistically significant compared with the control value without [6]-gingerol or lafutidine. [b]$P<0.01$, statistically significant compared with the capsaicin-induced contraction in the control ileum.

($P<0.05$). The submaximal contraction induced by 100 nM ACh (Fig. 1, trace h) and the maximal contraction induced by 3 μM ACh in the 1 μM lafutidine-treated ileum were almost the same as those in the control ileum (n = 4). In the 1 mM lafutidine-treated ileum, the submaximal response by 100 nM ACh was slightly (30 – 40%) inhibited, and the maximal response by 3 μM ACh was significantly but slightly inhibited (82.3 ± 4.0%, n = 4, $P<0.05$). Famotidine, another antagonist of histamine H_2 receptor, at all concentrations tested (100 nM – 1 mM), showed no effect on the contraction of the guinea pig ileum alone or in combination with 3 μM capsaicin; and the contraction induced by 3 μM ACh was significantly but slightly inhibited in the 1 mM famotidine-treated ileum (78.0 ± 5.2%, n = 4, $P<0.05$).

Effects of capsaicin, [6]-gingerol, and lafutidine on [^{3}H]choline release from the prelabeled slices of guinea pig ileum

The addition of 30 μM capsaicin or 30 μM [6]-gingerol stimulated [^{3}H]choline (and [^{3}H]ACh) release from the prelabeled slices of the ileum in the presence of 2 mM $CaCl_2$ (Table 3). The [^{3}H]choline releases induced by capsaicin and [6]-gingerol in the absence of extracellular $CaCl_2$ were markedly less than those in the presence of 2 mM $CaCl_2$ (data not shown, n = 4). The release induced by co-addition of 30 μM capsaicin and 30 μM [6]-gingerol was significant compared with the control, but was not additive by the two stimulants. Lafutidine (10 μM – 1 mM) alone had no effect on [^{3}H]choline release. Co-addition of 30 μM lafutidine inhibited 30 μM capsaicin-induced [^{3}H]choline release. Because of wide variations, we could not detect significant effects of the agents at low concentrations and could not study the effect of pretreatment with [6]-gingerol or lafutidine on 3 μM capsaicin-induced [^{3}H]choline release.

Table 1. Effects of capsazepine, ruthenium red, atropine, and tetrodotoxin on capsaicin- and [6]-gingerol-induced contraction in the vehicle-, [6]-gingerol-, or lafutidine-treated ileum

Treatment	Vehicle-treated	Vehicle-treated	[6]-Gingerol-treated	Lafutidine-treated
Addition	3 μM Capsaicin	30 μM [6]-Gingerol	3 μM Capsaicin	3 μM Capsaicin
	Contraction (% of ACh response)			
None	20.3 ± 2.4	13.3 ± 2.8	47.6 ± 1.4[a]	38.3 ± 4.2[a]
Capsazepine	5.1 ± 3.6[b]	1.1 ± 0.3[b]	3.1 ± 1.8[b]	0.8 ± 0.7[b]
Ruthenium red	8.7 ± 3.3[b]	0.2 ± 0.3[b]	9.4 ± 8.0[b]	4.5 ± 2.6[b]
Atropine	4.3 ± 1.7[b]	0.2 ± 0.4[b]	3.2 ± 1.7[b]	0.8 ± 0.5[b]
Tetrodotoxin	0.6 ± 0.5[b]	0.1 ± 0.3[b]	1.1 ± 0.4[b]	2.3 ± 2.3[b]

The guinea pig ileum segments were incubated for 7 min with vehicle (None), 10 μM capsazepine, 10 μM ruthenium red, 1 μM atropine, or 100 nM tetrodotoxin. Then 3 μM capsaicin or 30 μM [6]-gingerol were added to measure contraction. In some experiments, the segments were incubated with 0.3 μM [6]-gingerol or 1 μM lafutidine for 7 min, and then the indicated concentrations of antagonists or inhibitor were added and further incubated for additional 7 min. Then 3 μM capsaicin was added to measure contraction. Values are each the mean ± S.E.M. for 4 – 6 independent animals. [a]$P<0.05$, statistically significant compared with the capsaicin-induced contraction in the vehicle-treated ileum. [b]$P<0.05$, statistically significant compared with the value without the indicated antagonists or inhibitor (None).

Table 2. Effects of indomethacin, hexamethonium, and CP96345 on capsaicin- and [6]-gingerol-induced contraction

Addition	Capsaicin	[6]-Gingerol	Substance P
	Contraction (% of ACh response)		
Experiment I			
Vehicle (None)	24.3 ± 4.3	20.8 ± 5.4	Not determined
Indomethacin	22.8 ± 3.6	19.8 ± 3.6	Not determined
Experiment II			
Vehicle (None)	23.3 ± 3.8	21.8 ± 4.5	20.2 ± 4.2
Hexamethonium	18.8 ± 3.4	19.9 ± 3.8	Not determined
CP96345	17.5 ± 2.8	18.7 ± 3.2	1.8 ± 1.2*

In Experiment I, the guinea pig ileum segments were incubated for 40 min with vehicle (None) or 20 μM indomethacin. In Experiment II, the segments were incubated for 10 min with vehicle, 100 μM hexamethonium, or 2 μM CP96345. Then 3 μM capsaicin, 100 μM [6]-gingerol, or 1 nM substance P was added to measure contraction. Values are each the mean ± S.E.M. for 3 – 4 independent animals. *$P<0.05$, statistically significant compared with the value without CP96345.

Table 3. Effects of capsaicin, [6]-gingerol, and lafutidine on [^{3}H]choline release from the prelabeled slices of guinea pig ileum

	[^{3}H]Choline release (% of total)	
Addition	None	30 μM Capsaicin
None	10.0 ± 1.4	13.9 ± 0.7*
30 μM [6]-Gingerol	14.9 ± 0.8*	16.0 ± 0.5*
30 μM Lafutidine	10.4 ± 2.0	10.7 ± 0.5^b

The prelabeled slices were incubated with vehicle (None), 30 μM capsaicin, 30 μM [6]-gingerol, or 30 μM lafutidine for 10 min at 37°C. Values were calculated as percentages relative to the total incorporation of [^{3}H]choline into the slices. Values are each the mean ± S.E.M. for 3 independent animals. *$P<0.05$, statistically significant compared with the control value (None). $^bP<0.05$, statistically significant compared with the value with capsaicin.

Discussion

Involvement of capsaicin-sensitive neurons on [6]-gingerol-induced contraction of guinea pig ileum

In the present study, we found that [6]-gingerol induced contraction alone and also enhanced the capsaicin-induced contraction in guinea pig ileum. The effects of [6]-gingerol appeared to be mediated by activation of vanilloid receptors on the neurons in the ileum. First, the effects of [6]-gingerol were almost completely inhibited by capsazepine and ruthenium red. Although these agents were reported to have non-specific actions such as inhibition of Ca^{2+} channels (30, 31), these agents at 10 μM markedly inhibited capsaicin- and [6]-gingerol-induced contractions without changing the ACh response. Second, the effect of [6]-gingerol was desensitized easily. Third, treatment of the ileum with [6]-gingerol at lower concentrations up to 0.3 μM enhanced the 3 μM capsaicin-induced contraction, and the enhanced contraction was eliminated by antagonists of vanilloid receptors. Fourth, treatment with higher concentrations greater than 3 μM of [6]-gingerol inhibited the capsaicin-induced contraction. [6]-Gingerol at any concentrations barely modified the contraction induced by activation of muscarinic ACh receptors on smooth muscles in the ileum. There is some evidence that capsaicin and zingerone can activate the same receptor and/or a common pathway on the afferent neurons in vivo and on the trigeminal ganglion neurons in vitro (15 – 17). Although [6]-gingerol and zingerone structurally differ from capsaicin in that the two agents have a shorter hydrophobic moiety and lack an acyl-amide moiety, both have a vanillyl-like moiety as is seen in the structure of capsaicin. Thus, it is probable that [6]-gingerol activates the capsaicin-sensitive vanilloid receptor.

Barthó and Vizi (6) reported that activation of vanilloid receptors with capsaicin stimulated the release of ^{3}H radioactivity from the myenteric plexus-longitudinal muscle preparation of the guinea pig ileum preincubated with [^{3}H]choline. In the present study, not only capsaicin but also [6]-gingerol stimulated [^{3}H]choline release from the prelabeled slices of the ileum (Table 3). In addition, treatment with atropine or tetrodotoxin almost completely inhibited not only capsaicin- but also [6]-gingerol-induced contractions (Table 1). These findings suggest that the contraction induced by [6]-gingerol were mediated by activation of vanilloid receptor and ACh release in the neurons and the following activation of muscarinic ACh receptors in the ileum.

Involvement of NK_1 receptors and prostaglandins formation on [6]-gingerol-induced contraction

Treatment with 100 μM hexamethonium had no effect on capsaicin- and [6]-gingerol-induced contraction in the guinea pig ileum. Like capsaicin (7), the effect of [6]-gingerol did not appear to be mediated by nicotinic ganglionic neurons. The capsaicin-induced contractions in many gastrointestinal preparations are reported to be mediated through the release of neuropeptides such as neurokinins from capsaicin-sensitive neuronal structures (9, 32, 33). Barthó et al. (5) reported that the contraction evoked by capsaicin in the ileum was not reduced when NK1 or NK3 receptors were blocked separately, whereas a combined blockade of NK receptors significantly depressed the capsaicin response. Although an antagonist of the NK_1 receptor alone had no effect on capsaicin- and [6]-gingerol-induced contraction in the present study, we could not exclude the involvement of other

NK receptors in the contractile responses. In addition, it should be determined whether ACh is released from the neurons having VR1 directly and/or through the possible involvement of other inter-neurons having neurokinins and other mediators.

[6]-Gingerol potentiated prostaglandins ($F_{2\alpha}$, E_2 and I_2)-induced contractions in mouse mesenteric veins, although [6]-gingerol alone showed no effect (10, 11). Since it has been established that stimulation of prostanoid receptors caused contraction in the guinea pig ileum (34), prostaglandin-like compounds may be involved in [6]-gingerol-induced contraction. However, [6]-gingerol is reported to be an inhibitor of prostaglandin synthetase and 5-lipoxygenase in vitro (35, 36). As treatment with indomethacin did not modify the contraction induced by [6]-gingerol, the effects of [6]-gingerol did not appear to be due to prostaglandin(s) formation.

Possible interaction to capsaicin-sensitive neurons by lafutidine

Lafutidine is a new type antagonist of histamine H_2 receptors (18, 19). Several studies including ours suggested that the capsaicin-sensitive neurons are involved in the gastro-protection induced by lafutidine in vivo (19 – 22). In the present study, treatment of the isolated ileum preparation with 1 μM lafutidine, which alone showed no effect, enhanced the 3 μM capsaicin-induced contraction. The enhancement of the capsaicin response induced by lafutidine was abolished by tetrodotoxin and by atropine. Onodera et al. (19) also reported that the gastro-protective effect of lafutidine in rats was decreased by intra-arterial infusion of tetrodotoxin in vivo. Treatment with lafutidine at higher concentrations (100 μM and 1 mM) showed a phenomena similar to desensitization; inhibition of the contraction induced by 3 μM capsaicin. Famotidine had no effect on the capsaicin response. The addition of 30 μM lafutidine alone did not stimulate [^{3}H]choline release from the prelabeled slices of the ileum, but co-addition of lafutidine inhibited the capsaicin-induced [^{3}H]choline release. Although the reasons are not clear at present, this observation may be in relation to the desensitization. The present study is the first to report the involvement of the capsaicin-sensitive neurons and/or pathway on the response induced by lafutidine in vitro (in isolated preparation), to our knowledge.

Summary and problems to be determined

Recently, Dedov et al. (37) reported that [6]-gingerol evoked intracellular Ca^{2+} transients in rat dorsal root ganglion neurons via VR1. In the present study, [6]-gingerol at concentrations greater than 30 μM alone induced contraction of the ileum via activation of vanilloid receptors. Treatment with [6]-gingerol or lafutidine depending on their concentrations showed dual effects on capsaicin-induced contraction of the ileum: stimulation at lower concentrations and inhibition at higher concentrations. Our conclusion is that both [6]-gingerol and lafutidine have some interactions with capsaicin-sensitive neurons and/or vanilloid receptors and thus regulate the contraction via ACh release in isolated guinea pig ileum in vitro.

Pharmacological evidence suggested the existence of vanilloid receptor subtypes with distinct characteristics (38), and a homolog of VR1 was cloned (39). [6]-Gingerol and lafutidine may interact with these putative homologs of VR1. Specifically, the molecular mechanism clarifying how lafutidine regulates the capsaicin-sensitive pathway remains to be clarified. Some natural products, which are used extensively in indigenous medicine, are proposed as novel-type vanilloid receptor agonists, and these compounds may be useful for development of ligands of vanilloid receptors (40). Further studies on screening and identification of natural products and/or endogenous compounds that can regulate the functions of vanilloid receptors are currently in progress in our laboratory.

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